



## INDUCTION OF CROSS-REACTIVE ANTIBODIES AGAINST A SELF PROTEIN BY IMMUNIZATION WITH A MODIFIED SELF PROTEIN CONTAINING A FOREIGN T HELPER EPITOPE

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**Abstract**—Self proteins are handled in the same way as foreign proteins by antigen presenting cells, but because of T-cell tolerance the presentation of self peptides does not normally lead to T cell activation. By providing physically linked T-cell help it is possible to overcome the B cell non-responsiveness toward self antigens. We have shown previously that a very potent antibody response, cross-reactive with a self protein, can be rapidly induced by immunizing with a recombinant immunogen consisting of the self protein with a foreign immunodominant T helper epitope inserted into its sequence (Dalum, I., Jensen, M. R., Hindersson, P., Elsner, H. I. and Mouritsen, S. (1996) *J. Immunol.* 157, 4796). In this study we compare this approach for inducing autoantibodies against a self protein with the traditional method of conjugating the self antigen to a foreign carrier protein. The highly conserved self protein ubiquitin with an inserted epitope from ovalbumin (UbiOVA) is used as a model protein and compared to two traditionally conjugated immunogens consisting of ubiquitin chemically conjugated to a peptidic T helper epitope or to ovalbumin. The traditionally conjugated immunogens induce much slower and low titered ubiquitin specific antibody responses than the recombinant construct which also is capable of inducing antibodies directed against a much broader range of potential ubiquitin B cell determinants than the chemically conjugated immunogens. All three constructs are processed by antigen presenting cells and ovalbumin derived T cell epitopes are presented to T helper cells. From these observations it seems likely that the presence of non-shielded autologous B cell determinants on the immunogen is critical for the ability to induce a strong autoantibody response with a diverse fine specificity. Furthermore, the ubiquitin specific antibodies induced by UbiOVA contain higher levels of IgG2a/b relative to IgG1 compared to the conjugates. We therefore speculate that the insertion of a T cell epitope directly into the self antigen could possibly induce an immune response with a different Th1/Th2 balance than a response induced with traditional conjugates. © 1997 Elsevier Science Ltd. All rights reserved.

**Key words:** Self antigen, T helper epitope, B cell tolerance, autoantibodies, conjugated antigens.

### INTRODUCTION

In order to be recognized by T helper (Th) cells, antigens (Ags) must be processed by antigen presenting cells which proteolytically process the Ags to generate peptide fragments which are subsequently exposed on the cell surface in complex with MHC class II molecules. B cells

can act as highly potent antigen presenting cells when they endocytose Ags via their specific surface immunoglobulin receptors (Rock *et al.*, 1984). The subsequent Th cell recognition of peptides presented by MHC class II on the surface of B cells leads to direct T-cell help to the B cell and eventually to generation of antibodies (Abs) against the intact Ag.

Peptides from self proteins are presented by antigen presenting cells in the same way as peptides generated by Ag processing of foreign proteins (Kourilsky *et al.*, 1987). Normally, the presentation of self peptides does not lead to stimulation of T cells because of tolerance toward MHC associated epitopes derived from the self proteins. A major reason for the non-responsiveness of self reactive B cells could be the lack of direct T-cell help. Accordingly, to make self Ags immunogenic, immunostimulatory Th epitopes have been coupled chemically to the Ags (Talwar

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**Abbreviations:** Ab, antibody; Ag, antigen; UbiOVA, recombinant ubiquitin with OVA(325-336) inserted at position 21-32; cUbi-OVAp, bovine ubiquitin chemically conjugated with OVA(323-339)Y peptide; cUbi-OVA, bovine ubiquitin chemically conjugated to ovalbumin; HEL, hen egg-white lysozyme; OVA, ovalbumin.

*et al.*, 1994; Sad *et al.*, 1993; Steinhoff *et al.* 1994). However, this often leads to low-titered and slowly induced autoantibody responses. One explanation for this could be that autologous B cell epitopes in the immunogen are physically shielded by the relatively large carrier moieties. Another possibility could be that chemical conjugates are less susceptible to intracellular Ag processing than non-conjugated proteins and therefore lead to an impaired presentation of the conjugated Th epitopes.

We have previously studied the breakdown of the B cell tolerance against the non-immunogenic self Ag ubiquitin of which the amino acid sequence is totally conserved among the animal species. This protein was modified by insertion of single foreign immunodominant Th cell epitopes (Dalum *et al.*, 1996). We showed that the cross-reactive ubiquitin-specific autoantibody response raised toward such recombinant immunogens was very strong and rapidly induced, thereby demonstrating that the T-cell help provided by insertion of a single foreign Th epitope is sufficient to overcome the B cell non-responsiveness. The T cell regulatory role of such an inserted foreign Th epitope was studied and at least two different Th cell specificities (against the inserted epitope as well as against novel flanking epitopes) were found to operate during the Ab response (Dalum *et al.*, 1996). Here, we directly compare this approach with a traditional chemical conjugation method commonly used to create hapten-carrier complexes. We examine the ability of a well-defined foreign immunodominant core Th cell epitope OVA(325–336) (Sette *et al.*, 1987) to induce T-cell help for autoantibody production when linked in different ways to the same self protein. The recombinant construct, UbiOVA, consists of ubiquitin with OVA(325–336) inserted at position 21–32. The conjugates cUbi-OVAp and cUbi-OVA were produced by chemical conjugation of the OVA(323–339)Y peptide or OVA, respectively, to bovine ubiquitin using a standard method. We demonstrate that the recombinant approach is superior to the classical conjugation method with regard to inducing specific high-titered cross-reactive autoantibodies. It is also shown that the recombinant immunogen is able to induce autoantibodies with a more diverse fine specificity than the conjugates. A possible effect on the Th1/Th2 balance of the response induced against the recombinant immunogen is also indicated.

## MATERIALS AND METHODS

### Immunogens

UbiOVA was constructed as previously described (Dalum *et al.*, 1996). Briefly, a segment corresponding to amino acids 21–32 of a synthetic gene encoding ubiquitin was exchanged with oligonucleotides encoding the Th epitope OVA(325–336) (QAVHAAHAEINE) using conventional techniques (Sambrook *et al.*, 1989). The modified protein was purified from *E. coli* inclusion bodies using urea solubilization and ammonium sulphate precipitation as well as reversed phase HPLC. The resulting

material was more than 95% pure as determined on silver-stained SDS-PAGE gels. The protein concentration was determined by the bicinchoninic acid method (Smith *et al.*, 1985) and the material was solubilized in phosphate-buffered saline (PBS) (0.15 M NaCl, 2.5 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2) and stored at –20°C until use. UbiHEL was constructed by substitution of ubiquitin amino acids 65–76 with an amino acid sequence corresponding to the T cell epitope 50–61 of hen egg-white lysozyme (HEL) (STDYGILQINSR) and the resulting protein was expressed and purified in the same way as UbiOVA (Dalum *et al.*, 1996).

The conjugates cUbi-OVAp and cUbi-OVA were produced by conventional two-step glutaraldehyde coupling (Harlow and Lane, 1988). Briefly, 5 mg of bovine ubiquitin (Sigma, Dorset, U.K.) was dissolved in 500 µl PBS and stirred at room temperature with 500 µl 10% glutaraldehyde for 30 min, and subsequently glycerol was added to 5%. Activated ubiquitin was separated from free glutaraldehyde by collection of OD=280 absorbing material eluted in the void volume of a gel filtration column (G25 superfine, Pharmacia, Uppsala, Sweden) equilibrated in PBS. Synthetic OVA(323–339)Y (1.77 mg dissolved in 1 ml PBS) or OVA (grade VII, Sigma, 3.37 mg dissolved in 1 ml PBS) were added and incubated with stirring at room temperature for 2 h, the last hour in the presence of 200 mM glycine. The material was dialysed against PBS and the protein content was determined as before (Smith *et al.*, 1985). The conjugates were stored at –20°C until use.

### Synthetic peptides

Synthetic peptides representing ubiquitin(1–15) (MQIFVKTLTGKTITL), ubiquitin(11–25) (KTITLEVEPSDTIEN), ubiquitin(21–36) (DTIENVKAKIQDKEGI), ubiquitin(32–46) (DKEGIPPDQQLIFA), ubiquitin(42–56) (RLIFAGKQLEDGRTL), ubiquitin(52–66) (DGRTLSDYNIQKEST), ubiquitin(62–76) (QKESTLHLVLRRLRGG), UbiOVA(19–34) (PSQAVHAAHAEINEKE) and OVA(323–339)Y (ISQAVHAAHAEINEAGRY) were synthesized on an automatic peptide synthesizer (Novasyn Crystal, Novasyn, Nottingham, U.K.) using conventional Fmoc chemistry on a cleavable resin (Tentagel, Rapp Polymere, Tübingen, Germany). PyBOP (Novasyn) was used for activation of the amino acids. The peptides were cleaved from the resins and deprotected according to the manufacturers instructions, dissolved in 10% acetic acid and lyophilized before purification by reversed phase HPLC using a LiChrosorb RP-18 column (Merck, Darmstadt, Germany). A high degree of purity of all the peptides was verified by analytical reversed phase HPLC. The sequences of all peptides were subsequently confirmed using an automatic peptide sequencer (model 476A, Applied Biosystems, Foster City, CA, U.S.A.).

### Induction of ubiquitin specific Abs

BALB/c (H-2<sup>d</sup>) mice were bred at authorized facilities at the Panum Institute, Copenhagen, Denmark. Five

groups of 8 animals were immunized with either UbiOVA, cUbi-OVA, cUbi-OVAp, bovine ubiquitin or bovine ubiquitin mixed with OVA(323-339)Y. One group of five animals received UbiHEL. The animals were immunized at day 1 by subcutaneous (s.c.) injections of 100  $\mu$ g Ag solubilized in PBS and emulsified with an equal volume of complete Freund's adjuvant (CFA) (Difco, Detroit, MI, U.S.A.). In the group receiving a mixture of ubiquitin and OVA(323-339)Y, 100  $\mu$ g of each of the Ags were given. Booster injections with the same amount of Ag emulsified 1:1 in incomplete Freund's adjuvant (Difco) were given s.c. on days 14 and 28. Each mouse was bled by puncture of the orbital plexus at days 0, 13, 27, 41, 55, 69 and 102, and sera were isolated by centrifugation and stored at  $-20^{\circ}\text{C}$  until use.

### ELISA

For detection of ubiquitin specific Abs, polystyrene microtitre plates (Maxisorp, Nunc A/S, Roskilde, Denmark) were coated overnight (100  $\mu$ l/well) at  $4^{\circ}\text{C}$  with yeast ubiquitin (50  $\mu$ g/ml) (Sigma) dissolved in 0.1 M sodium carbonate buffer (pH 9.6). Yeast ubiquitin differs from bovine ubiquitin only at three amino acid positions (Özkaynak *et al.*, 1984) but was used in order to avoid any possible cross-reactions with Abs specific for impurities in the bovine ubiquitin preparation used to generate the conjugates. Residual binding sites were blocked with 200  $\mu$ l/well of 1% BSA in washing buffer (0.5 M NaCl, 2.5 mM KCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , 7 mM  $\text{Na}_2\text{HPO}_4$ , 1% Triton X-100, pH 7.2), and 3-fold dilutions of serum (100  $\mu$ l/well in washing buffer containing 1% BSA) starting at 1:200 were added to the coated wells and incubated for 1 h at room temperature. After washing, 100  $\mu$ l of horse radish peroxidase labelled rabbit anti-mouse immunoglobulin (DAKO A/S, Ejby, Denmark) diluted 1:2000 in washing buffer containing 1% BSA was added to each well and incubated for 1 h at room temperature. The binding was subsequently visualized with a 1 mg/ml *o*-phenylenediamine substrate (Sigma) solution in 3.5 mM citrate, 7.5 mM  $\text{Na}_2\text{HPO}_4$  (pH 5.0) containing 0.03%  $\text{H}_2\text{O}_2$ . The reaction was stopped by addition of 150  $\mu$ l 2 N  $\text{H}_2\text{SO}_4$  per well, and the absorbance at 492 nm was measured. A standard curve with known dilutions of high-titered anti-UbiOVA antiserum was included on each plate. The titers are expressed as the dilution given by the standard curve which corresponds to the observed absorbance of the serum sample. Data are represented by calculating the mean of the titers within each immunization group. The reactivity of the antisera with OVA protein was determined in a similar assay with polystyrene plates coated with 1  $\mu$ g/well of OVA. The ability of antibodies raised against ubiquitin or OVA to react with the different immunogens was tested by coating the individual immunogens (5  $\mu$ g/well) in Maxisorp microtiter plates and performing the assay as described above. A polyclonal anti-ubiquitin antiserum raised against native ubiquitin conjugated to bovine IgG (generously supplied by Klaus Hendil, The August Krogh Institute, University of Copenhagen,

Denmark) and a polyclonal anti-OVA antiserum raised against OVA, coupled to dinitrophenyl, were used as primary antibodies. Horse radish peroxidase labelled swine anti-rabbit immunoglobulin (DAKO) was used as secondary antibody in the anti-OVA reactions. The fine specificities of the antisera were determined in an ELISA assay using AquaBind microtitre plates (M&E Biotech, Copenhagen, Denmark) which are capable of binding peptides covalently in a hydrophilic environment (Gregorius *et al.*, 1995). These plates were coated for 2 h at  $37^{\circ}\text{C}$  with 10  $\mu$ g/ml synthetic ubiquitin peptide dissolved in 0.1 M sodium carbonate buffer (pH 9.6) (100  $\mu$ l/well). The plates were subsequently blocked with 200  $\mu$ l/well of 0.1 M sodium carbonate buffer (pH 9.6) containing 10 mM ethanolamine, 15% polyethylene glycol 8000 (Sigma) and 1% BSA. Serial dilutions of pooled sera from each of the four ubiquitin-reactive immunization groups were made in washing buffer containing 1% BSA and 1% dextran 70,000 (Sigma) and allowed to react at room temperature for 2 h (100  $\mu$ l in each well). After washing, 100  $\mu$ l of alkaline phosphatase labelled rabbit anti-mouse immunoglobulin diluted 1:500 in washing buffer containing 1% BSA was added to each well and incubated for 2 h at room temperature. The binding was visualized with 100  $\mu$ l of a 1 mg/ml *p*-nitrophenylphosphate substrate solution in 1 M diethanolamine, 0.5 mM  $\text{MgCl}_2$  (pH 10.0), and the absorbance at 405 nm was measured. The reactivity of the sera was compared using the obtained  $A_{405}$  values at a selected representative dilution (1:100). Isotyping analysis was performed on pools of ubiquitin-reactive sera using a standard kit (90-6550, Zymed, San Francisco, CA, U.S.A.) with 5  $\mu$ g bovine ubiquitin per well for coating.

### T cell proliferation assay

This was done as described previously (Mouritsen *et al.*, 1989). Groups of 2 BALB/c mice were primed s.c. in the hind footpads and at the tail base with 100  $\mu$ g Ag dissolved in PBS emulsified 1:1 in CFA. After 10 days popliteal, inguinal and periaortal lymph nodes were removed and single-cell suspensions prepared.  $5 \times 10^5$  lymph node cells were mixed with Ag in a total volume of 200  $\mu$ l/well of complete RPMI-1640 medium containing 1 mM sodium pyruvate, MEM non-essential amino acids and 0.5-1% fresh syngeneic normal mouse serum. Concanavalin A (1  $\mu$ g/ml) as well as purified protein derivative of tuberculin (50  $\mu$ g/ml) were used as positive controls. The assay was performed in triplicate in wells of microtitre plates. After an 18 h pulse with 1  $\mu\text{Ci}$   $^3\text{H}$ -thymidine per well at the end of a 4 day incubation period, the proliferative response was measured on harvested cells in a  $\beta$ -scintillation counter. At least three repetitions of the assay were performed with equivalent results. Data were presented as T cell proliferation indices calculated as the  $^3\text{H}$ -thymidine incorporation of cells cultured with Ag relative to the  $^3\text{H}$ -thymidine incorporation of cells cultured with PBS.

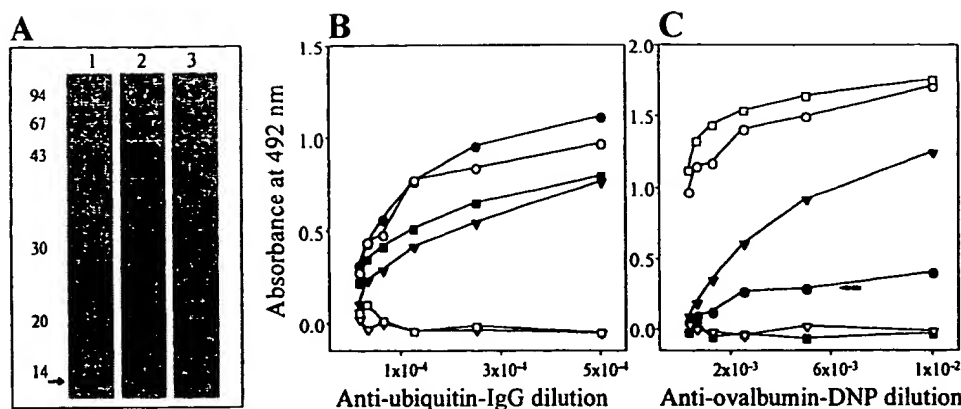


Fig. 1. Characterization of the immunogens. (A) Samples of 5  $\mu$ g (1) UbiOVA, (2) cUbi-OVA and (3) cUbi-OVAp were applied in lanes of an SDS-PAGE gel, and the proteins were visualized by silver staining. The migration of the native 8.5 kDa ubiquitin protein is indicated by an arrow. Polystyrene ELISA plates were coated with ( $\blacktriangledown$ ) UbiOVA, ( $\bullet$ ) cUbi-OVAp, ( $\circ$ ) cUbi-OVA, ( $\blacksquare$ ) bovine ubiquitin, ( $\square$ ) OVA or ( $\nabla$ ) OVA(323–339)Y peptide and tested for reactivity with (B) antiserum raised against ubiquitin coupled to bovine IgG and (C) antiserum raised against OVA coupled to dinitrophenyl (DNP).

## RESULTS

### *Production of the immunogens UbiOVA, cUbi-OVAp and cUbi-OVA*

To address whether insertion of a single Th epitope is more efficient than chemical conjugation in order to induce Abs against a self protein, three different immunogens were produced, all containing the same core T cell epitope derived from OVA. Figure 1A shows the immunogens visualized in a silver stained SDS-PAGE gel. The immunogens could be recognized in Western blots both using polyclonal anti-ubiquitin antiserum raised against a conjugate of the native ubiquitin molecule and bovine IgG as well as with a polyclonal anti-OVA antiserum raised against OVA coupled to dinitrophenyl (data not shown). From the smeared appearance of the protein bands on the Western blots it was concluded that the conjugation procedure results in quite heterogenous preparations of both cUbi-OVA and cUbi-OVAp. The presence of both ubiquitin and OVA derived determinants in all three immunogens was also verified in an ELISA assay using the same antisera (Fig. 1B and 1C).

### *Immunogenicity of UbiOVA, cUbi-OVAp and cUbi-OVA*

Five groups of 8 BALB/c mice were immunized s.c. with either UbiOVA, cUbi-OVAp, cUbi-OVA, bovine ubiquitin mixed with OVA(323–339)Y or bovine ubiquitin alone. The reactivity of the antisera toward non-conjugated ubiquitin was analysed in ELISA with immobilized yeast ubiquitin (Fig. 2). All mice receiving UbiOVA rapidly developed a high-titered anti-ubiquitin response, confirming our previous observation that insertion of the OVA(325–336) epitope into ubiquitin results in a highly immunogenic molecule capable of inducing Abs cross-reactive with non-modified ubiquitin (Dalum *et al.*, 1996). Immunization with the cUbi-OVAp con-

jugate resulted in a much slower and low-titered anti-ubiquitin response which appeared in only 3 out of 8 mice. A slow low-titered response was also observed in the group of mice immunized with the cUbi-OVA conjugate, although 7 out of 8 mice developed anti-ubiquitin Abs. Strikingly, the anti-ubiquitin titers of the Abs raised

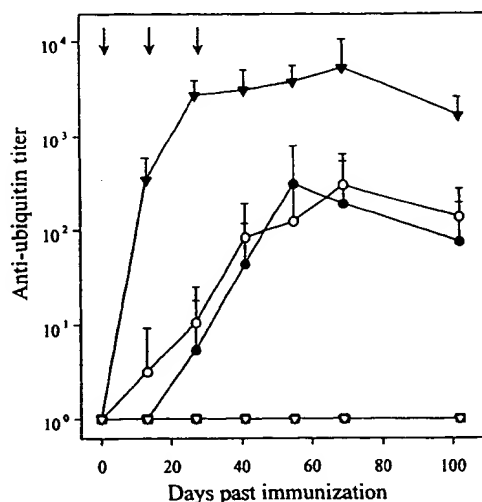


Fig. 2. Induction of anti-ubiquitin Abs in sera from BALB/c mice immunized with ( $\blacktriangledown$ ) UbiOVA, ( $\bullet$ ) cUbi-OVAp conjugate or ( $\circ$ ) cUbi-OVA conjugate. Control mice were immunized with ( $\blacksquare$ ) bovine ubiquitin alone or ( $\nabla$ ) mixed with OVA(323–339)Y peptide. Groups of 8 mice received s.c. injections of 100  $\mu$ g Ag emulsified 1:1 in CFA on day 1. Booster injections with the same amount of Ags in incomplete Freund's adjuvant were given s.c. on days 14 and 28. The immunization dates are indicated with arrows. Each mouse was bled at various time points, and the obtained sera were analysed in ELISA for anti-ubiquitin reactivity. The titer was calculated by correlation with a strongly positive standard serum. Error bars represent one positive standard deviation.

toward UbiOVA were approximately  $20\times$  higher than the Abs raised with the conjugates. Furthermore, a detectable level of anti-ubiquitin Abs was induced approximately two weeks earlier with UbiOVA as immunogen than with the conjugates. Native bovine ubiquitin alone or mixed with OVA(323–339)Y peptide did not induce ubiquitin specific Abs. In a separate ELISA assay, the sera were tested for their ability to react with OVA. All mice immunized with UbiOVA, cUbi-OVA or bovine ubiquitin mixed with OVA(323–339)Y peptide developed anti-OVA Abs, whereas sera from 5 out of 8 mice immunized with cUbi-OVAp were reactive with OVA (data not shown).

#### Fine specificity of the induced autoantibodies

In order to compare the fine specificity of the Abs raised toward UbiOVA and the conjugates, 7 synthetic overlapping 15-mer peptides representing the entire ubiquitin sequence were synthesized. The peptides were immobilized in AquaBind microtitre plates, and pools of ubiquitin-reactive antisera raised in BALB/c mice toward UbiOVA and the conjugates were tested for reactivity with the peptides (Fig. 3). The antisera raised toward

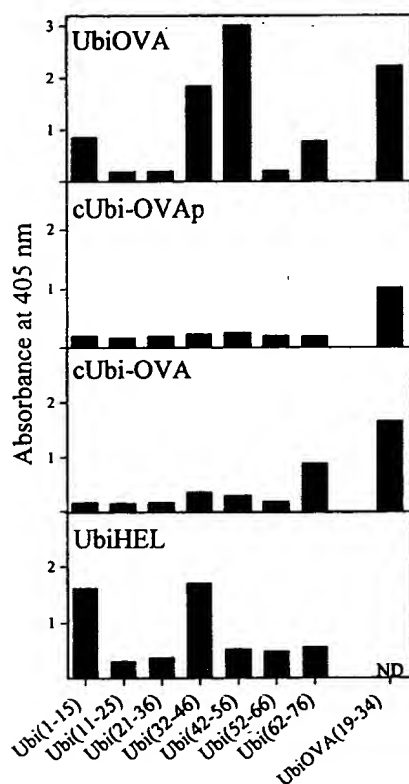


Fig. 3. Fine specificity of the Abs raised toward recombinant modified ubiquitin and ubiquitin conjugates. Pooled ubiquitin-reactive antisera raised in BALB/c mice toward UbiOVA, cUbi-OVAp, cUbi-OVA and UbiHEL were tested in ELISA for reactivity with peptides corresponding to overlapping ubiquitin sequences as well as the peptide UbiOVA(19–34) which corresponds to the OVA(325–336) epitope in UbiOVA with the two flanking ubiquitin amino acids in each end. ND: Not determined.

UbiOVA reacted with ubiquitin(32–46) and ubiquitin(42–56) and to a lower extent ubiquitin(1–15) and ubiquitin(62–76). The anti-cUbi-OVAp antisera did not react significantly with any of the overlapping peptides, whereas anti-cUbi-OVA antisera reacted exclusively with ubiquitin(62–76). Pooled immune sera from each of the three immunization groups reacted with bovine ubiquitin coated onto these microtitre plates as well as with the peptide UbiOVA(19–34) which corresponds to the OVA(323–339) derived OVA epitope in UbiOVA with two flanking ubiquitin amino acids in each end. Strong reactivity with ubiquitin(1–15) and ubiquitin(32–46) was observed when using another recombinant immunogen, UbiHEL which is constructed by inserting the H-2<sup>k</sup> restricted Th epitope HEL(50–61) into ubiquitin at position 65–76. It has previously been shown that the immunogenicity of such a modified recombinant self antigen in mice bearing non-matching MHC alleles can be due to the induction of a T-cell response specific for one or more epitopes composed of part foreign sequence and part ubiquitin sequence (Dalum *et al.*, 1996).

#### Isotype distribution of the induced autoantibodies

The relative occurrence of the different immunoglobulin isotypes was determined in pooled ubiquitin-reactive sera from the different groups of immunized mice. It was found that the antisera raised in response to all three immunogens were almost exclusively of the IgG isotype (Fig. 4). Compared to sera from mice immunized with the conjugates, the Abs raised against UbiOVA had a higher content of IgG2b (31% for UbiOVA compared to 5% and 9% for cUbi-OVAp and cUbi-OVA, respectively), whereas the level of IgG1 was lower (33% for UbiOVA compared to 56% and 45% for cUbi-OVAp and cUbi-OVA, respectively).

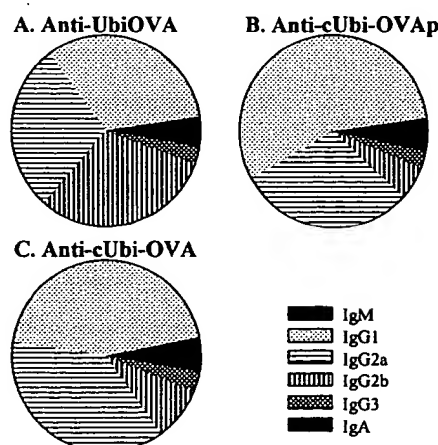


Fig. 4. Distribution of immunoglobulin isotypes in sera from mice immunized with UbiOVA or ubiquitin conjugates. Pooled ubiquitin-reactive sera from mice immunized with (A) UbiOVA, (B) cUbi-OVAp and (C) cUbi-OVA were analysed for the content of different immunoglobulin isotypes, and the result was illustrated as proportions of total anti-ubiquitin immunoglobulin heavy chain measured.

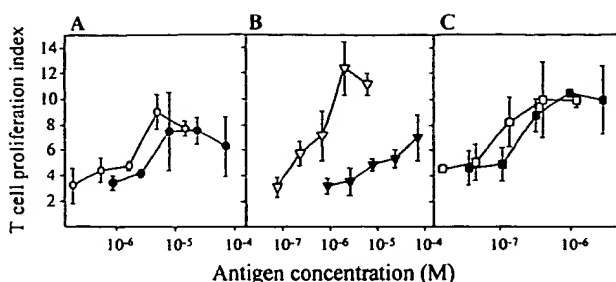


Fig. 5. Proliferation of T cells from BALB/c mice immunized with (A) UbiOVA(19-34), (B) OVA(323-339)Y or (C) OVA and *in vitro* stimulated with (○) UbiOVA, (▽) cUbi-OVAp or (□) cUbi-OVA, respectively. As control Ags (●) UbiOVA(19-34), (▼) OVA(323-339)Y or (■) OVA were added *in vitro*. BALB/c mice were immunized s.c. with 100 µg of Ag emulsified 1:1 in CFA. After 10 days lymph node cells were collected and incubated with serial dilutions of the respective Ags. T cell proliferation indices were determined after 4 days of culture. Average background levels were (A) 230 cpm, (B) 407 cpm and (C) 1325 cpm. Error bars represent one standard deviation.

#### *T cell responses against UbiOVA, cUbi-OVAp and cUbi-OVA*

To demonstrate that the added OVA epitope(s) in UbiOVA and the conjugates could be presented to T cells, we immunized 3 groups of BALB/c mice with any of the three OVA parts which were added in the three immunogens. As shown in Fig. 5, UbiOVA induced *in vitro* restimulation of T cells from mice immunized with UbiOVA(19-34) peptide. Likewise, cUbi-OVAp induced *in vitro* restimulation of T-cells raised against OVA(323-339)Y, and cUbi-OVA was able to restimulate T cells from mice immunized with OVA. As controls, all the T cells were restimulated with the Ag they were raised against. Thus, in all three immunogens, antigen presenting cells select the inserted or conjugated OVA epitope for presentation to Th cells raised in BALB/c mice.

## DISCUSSION

Normally, the self proteins of an individual are not attacked by specific autoantibodies. This tolerance within the B cell population is, by many immunologists, believed to be maintained by clonal deletion (Nemazee and Buerki, 1989) and/or by induction of anergy (Goodnow *et al.*, 1988) in the population of potentially autoreactive B cells. We have shown, however, that B cells reactive against self epitopes can be activated solely by providing strong foreign immunodominant Th epitopes to a self protein. By substitution of a ubiquitin segment with one such epitope we constructed a recombinant immunogen which is capable of inducing high-titered Abs reactive with native parts of the ubiquitin molecule. This approach was compared to immunogens produced by a classical chemical coupling method in terms of the dynamics of autoantibody induction, fine specificity of the induced Abs, IgG subclass distribution and avail-

ability of immunogenic T cell epitope(s) for presentation to Th cells.

We observed that the immunogen generated using the recombinant approach was able to induce a specific autoantibody response which was faster and with approximately 20-fold higher titers than the response induced with the traditional conjugates (Fig. 2). An explanation for this difference may be found in the structure of the immunogens. Ubiquitin is a small 76 amino acid protein which in addition to its N-terminal amino group contains seven lysine residues with amino groups that are potential coupling sites for carrier molecules when using the glutaraldehyde coupling method. However, no amino groups are located among the 13 carboxyl terminal amino acids. From crystallographic studies it is known that the carboxyl terminal is protruding from the otherwise very compact ubiquitin molecule (Vijay-Kumar *et al.*, 1987). It could thus be presumed that the ubiquitin part of the conjugates would be partly covered by the carrier moieties, but not in the carboxyl terminal. This is supported by the observation shown in Fig. 3, that the anti-ubiquitin Abs raised toward cUbi-OVA are mainly reactive with the carboxyl terminal peptide. We have also made the same observation using a ubiquitin conjugate with bovine IgG as the carrier molecule in rabbits (unpublished result). Our findings thus favour the explanation that physical shielding of native ubiquitin B cell epitopes except in the carboxyl terminal occurs in the ubiquitin-conjugates. Accordingly, this may be a major reason for the difference in capability of inducing anti-ubiquitin Abs using UbiOVA vs the classical conjugates. Another explanation for the weaker immunogenicity of the chemically linked conjugates could be that the ubiquitin moieties are seriously denatured during the conjugation procedure so that antibodies are induced towards denatured (and thus irrelevant) ubiquitin B cell epitopes assisted by an OVA specific Th cell response.

It has been reported that the use of large carrier molecules is associated with so-called carrier-induced suppression effects. Pre-immunization with the carrier protein or a derived T cell epitope has been found to reduce the Ab response to the carrier coupled Ag. This effect has by some workers been attributed to activation of carrier specific T suppressor cells (Sad *et al.*, 1991) whereas others explain such observations by competition between clonally expanded carrier specific B cells and hapten specific B cells (Schutze *et al.*, 1989). If carrier-induced suppression plays a role in breaking the B cell tolerance toward a self Ag, the use of an immunogen constructed like UbiOVA would perhaps circumvent these problems due to the use of a minimal part of the foreign carrier Ag in the construct.

Marked differences in the fine specificity distributions were observed among the recombinantly modified ubiquitin molecules and the conjugates. Even though only specificities against linear B cell epitopes represented by the peptides are measured, the fine specificities of the Abs raised against cUbi-OVAp and cUbi-OVA seemed less diverse than the Abs induced by the recombinant Ags

UbiOVA and UbiHEL (Fig. 3). This could perhaps be explained by the shielding of B cell epitopes in the conjugates, as mentioned above, and possibly also by the induction of antibodies against denatured ubiquitin determinants. In the design of UbiOVA we aimed at disturbing the tertiary ubiquitin structure as little as possible in order to preserve the native B cell epitopes. Therefore, we exchanged ubiquitin amino acids 21–32 which in the native context form an  $\alpha$ -helix (Vijay-Kumar *et al.*, 1987) with OVA(325–336) which can be modelled as an amphipathic  $\alpha$ -helix (Mouritsen *et al.*, 1991). In UbiHEL, HEL(50–61), which in itself does not form any known secondary structure, was substituted for the flexible carboxyl terminal ubiquitin segment 65–76. The inserted epitopes were not completely hidden, since Abs against the inserted foreign peptides were also present in the immune sera raised toward UbiOVA and UbiHEL (Dalum *et al.*, 1996).

In a vaccine, an ideal immunogen should induce an Ab response which is neutralizing in the sense that it eliminates or interferes with the biological effect of the target molecule in an appropriate way. An advantage of immunogens constructed by the insertion of a Th epitope into the target Ag is that they induce Abs with a more diverse fine specificity and thus may possess a better neutralizing ability. By comparing the results with UbiOVA and UbiHEL in Fig. 3, it can be seen that different epitopes inserted at different locations in ubiquitin gives rise to different autoantibodies to the self protein. We have also analysed a larger panel of modified self proteins with the foreign epitope inserted at several different positions. Immunization with these constructs give rise to high titered specific Abs with completely different fine specificities (yet unpublished result). This possibility of obtaining an Ab response with an appropriate fine specificity by selection of constructs with different insertion sites or different T cell epitopes may be an important advantage of this approach compared to classical conjugation.

We demonstrated that the immunoglobulins induced by the three immunogens were predominantly of the IgG subclass (Fig. 4), indicating that the autoantibody induction was obtained through a T cell dependent mechanism. In the case of UbiOVA we have previously shown that the induced immune response is T cell dependent. In BALB/c mice the T cell help was mediated through activation of Th cells specific for the inserted epitope (Dalum *et al.*, 1996). Here, we observed that the inserted or conjugated OVA epitope in all three immunogens could be presented to Th cells (Fig. 5). In other words, there is no apparent difference between the three immunogens concerning the availability of T cell immunogenic epitopes for Ag presentation. Thus, assuming that the observed T-cell responses quantitatively reflect the T-cell immunogenicity of the molecules, Ag presentation does not *a priori* seem to be a factor which can account for the observed difference in the Ab responses to the three immunogens. However, it does not exclude the possibility that the constructs are handled differently during the Ag processing in B cells.

In mice, both Th1 and Th2 cells are capable of providing effective help to B cells, although Th2 cells are usually more potent (Abbas *et al.*, 1996). It has been consistently found that Th1 helper cells preferentially induce secretion of Abs of the IgG2a and IgG2b isotypes whereas Th2 cells induce IgG1 and IgE responses (Abbas *et al.*, 1996; Stevens *et al.*, 1988). The ubiquitin specific IgG secretion observed in response to UbiOVA had a higher content of the IgG2b isotype relative to the IgG1 content than observed for the conjugated immunogens (Fig. 4). This suggests that using an immunogen constructed by inserting a single foreign immunodominant Th cell epitope such as in UbiOVA a deviation toward a Th1 dominated response can be obtained. It could therefore be speculated that the method which is used to provide linked T cell help can also affect the Th1/Th2 balance—in analogy to other proposed influential factors such as administration route, dosage and choice of adjuvant.

Generally, chemically linked conjugates can be technically difficult to define structurally. This may result in difficulties in characterizing and optimizing the antigens, batch-to-batch variations, poor reproducibility of experimental results and such conjugates may therefore have limited practical use in vaccines. By the use of recombinant immunogens produced as described in this work, some of these problems would be circumvented due to the more well-defined nature of the recombinant modified self-proteins.

In conclusion we found that insertion of a single foreign immunodominant Th epitope into a self protein in order to make the self Ag immunogenic have at least four major advantages over the traditional chemical conjugation method (1) the Ag is much more well defined, (2) cross-reactive autoantibodies are induced more rapidly and are more high-titered, (3) the induced autoantibodies have a more diverse fine specificity which can be varied depending both on the selected Th epitope and its intramolecular position, and (4) the possibility of the induced immune response having a different Th1/Th2 balance than a response induced with traditional conjugates.

Our results may have important therapeutic implications. In the treatment of different diseases where monoclonal Abs against pathogenic self proteins are used (Herzog *et al.*, 1989; Elliott *et al.*, 1993), vaccination against such proteins could be a feasible alternative. By the induction of endogenous Abs, severe inherent side effects of e.g. monoclonal antibodies such as the induction of allotypic or anti-idiotypic Abs (Elliott *et al.*, 1994) could be avoided. The principle of raising antibodies against pathogenic self proteins may be applicable to many chronic diseases (e.g. cancer and chronic inflammatory diseases) where an appropriate target protein can be identified. We are currently completing extensive studies which demonstrate the therapeutic ability of antibodies against a proinflammatory cytokine modified using this approach.

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## REFERENCES

- Abbas, A. K., Murphy, K. M. and Sher, A. (1996) Functional diversity of helper T lymphocytes. *Nature* **383**, 787–793.
- Dalum, I., Jensen, M. R., Hindersson, P., Elsner, H. I. and Mouritsen, S. (1996) Breaking of B cell tolerance toward a highly conserved self protein. *J. Immunol.* **157**, 4796–4804.
- Elliott, M. J., Maini, R. N., Feldmann, M., Long-Fox, A., Charles, P., Katsikis, P., Brennan, F. M., Walker, J., Bijl, H., Ghayeb, J. and Woody, J. N. (1993) Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor  $\alpha$ . *Arthritis Rheum.* **36**, 1681–1690.
- Elliott, M. J., Maini, R. N., Feldmann, M., Long-Fox, A., Charles, P., Bijl, H. and Woody, J. N. (1994) Repeated therapy with monoclonal antibody to tumour necrosis factor  $\alpha$  (cA2) in patients with rheumatoid arthritis. *Lancet* **344**, 1125–1127.
- Goodnow, C. C., Crosbie, J., Adelstein, S., Lavoie, T. B., Smith-Gill, S. J., Brink, R. A., Pritchard-Briscoe, H., Wotherspoon, J. S., Loblay, R. H., Raphael, K., Treni, R. J. and Basten, A. (1988) Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature* **334**, 676–682.
- Gregorius, K., Mouritsen, S. and Elsner, H. I. (1995) Hydrocoating: a new method for coupling biomolecules to solid phases. *J. Immunol. Methods* **181**, 65–73.
- Harlow, E. and Lane, D. (1988) *Antibodies. A laboratory manual*. p. 80. Cold Spring Harbor Laboratory, New York.
- Herzog, C., Walker, C., Müller W., Rieber, P., Reiter, C., Riethmüller G., Wassmer, P., Stockinger, H., Madic, O. and Pichler, W. J. (1989) Anti-CD4 antibody treatment of patients with rheumatoid arthritis: I. Effect on clinical course and circulating cells. *J. Autoimmun.* **2**, 627–642.
- Kourilsky, P., Chaouat, G., Rabourdin-Combe, C. and Claverie, J. (1987) Working principles in the immune system implied by the "peptidic self" model. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3400–3404.
- Mouritsen, S., Meldal, M., Rubin, B., Holm, A. and Werdelin, O. (1989) The T-lymphocyte proliferative response to synthetic peptide antigens of defined secondary structure. *Scand. J. Immunol.* **30**, 723–730.
- Mouritsen, S., Meldal, M., Ruud-Hansen, J. and Werdelin, O. (1991) T-helper-cell determinants in protein antigens are preferentially located in cysteine-rich antigen segments resistant to proteolytic cleavage by cathepsin B, L, and D. *Scand. J. Immunol.* **34**, 421–431.
- Nemazee, D. A. and Buerki, K. (1989) Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. *Nature* **337**, 562.
- Özkaynak, E., Finley, D. and Varshavsky, A. (1984) The yeast ubiquitin gene: head-to-tail repeats encoding a polyubiquitin precursor protein. *Nature* **312**, 663–666.
- Rock, K. L., Benacerraf, B. and Abbas, A. K. (1984) Antigen presentation by hapten-specific B lymphocytes. *J. Exp. Med.* **160**, 1102–1113.
- Sad, S., Gupta, H. M., Talwar, G. P. and Raghupathy, R. (1991) Carrier-induced suppression of the antibody response to a "self" hapten. *Immunology* **74**, 223–227.
- Sad, S., Chauhan, V. S., Arunan, K. and Raghupathy, R. (1993) Synthetic gonadotrophin-releasing hormone (GnRH) vaccines incorporating GnRH and synthetic T-helper epitopes. *Vaccine* **11**, 1145–1150.
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning. A laboratory manual*. Cold Spring Harbor Laboratory Press, New York.
- Schutze, M., Deriaud, E., Przewlocki, G. and Leclerc, C. (1989) Carrier-induced epitopic suppression is initiated through clonal dominance. *J. Immunol.* **142**, 2635–2640.
- Sette, A., Buus, S., Colon, S. M., Smith, J. A., Miles, C. and Grey, H. M. (1987) Structural characteristics of an antigen required for its interaction with Ia and recognition by T cells. *Nature* **328**, 395–399.
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76–85.
- Steinhoff, U., Burkhart, C., Arnheiter, H., Hengartner, H. and Zinkernagel, R. M. (1994) Virus or a hapten-carrier complex can activate autoreactive B cells by providing linked T help. *Eur. J. Immunol.* **24**, 773–776.
- Stevens, T. L., Bossie, A., Sanders, V. M., Fernandez-Botran, R., Coffman, R. L., Mosmann, T. R. and Vitetta, E. S. (1988) Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature* **334**, 255–258.
- Talwar, G. P., Singh, O., Pal, R., Chatterjee, N., Sahai, P., Dhall, K., Kaur, J., Das, S. K., Suri, S., Buckshee, K., Saraya, L. and Saxena, B. N. (1994) A vaccine that prevents pregnancy in women. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8532–8536.
- Vijay-Kumar, S., Bugg, C. E. and Cook, W. J. (1987) Structure of Ubiquitin refined at 1.8 Å resolution. *J. Mol. Biol.* **194**, 531–544.



## RESEARCH

# Therapeutic antibodies elicited by immunization against TNF- $\alpha$

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Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is critically involved in the pathogenesis of several chronic inflammatory diseases. Monoclonal antibodies against TNF- $\alpha$  are currently used for the treatment of rheumatoid arthritis and Crohn's disease. This report describes a simple and effective method for active immunization against self TNF- $\alpha$ . This vaccination approach leads to a T-cell-dependent polyclonal and sustainable anti-TNF- $\alpha$  autoantibody response that declines upon discontinuation of booster injections. The autoantibodies are elicited by injecting modified recombinant TNF- $\alpha$  molecules containing foreign immunodominant T-helper epitopes. In mice immunized with such molecules, the symptoms of experimental cachexia and type II collagen-induced arthritis are ameliorated. These results suggest that vaccination against TNF- $\alpha$  may be a useful approach for the treatment of rheumatoid arthritis and other chronic inflammatory diseases.

Keywords: TNF- $\alpha$ , vaccination, active immunization, autoantibodies, anti-TNF- $\alpha$ , collagen-induced arthritis (CIA), cachexia, chronic inflammatory disease

There is increasing evidence that the proinflammatory cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) plays a key role in the pathogenesis of chronic inflammation. It has been demonstrated in several animal models and clinical trials that suppression of TNF- $\alpha$  with either anti-TNF- $\alpha$  monoclonal antibodies or with soluble chimeric TNF- $\alpha$  receptors reduces the symptoms of inflammatory disease<sup>1-7</sup>. However, monoclonal antibodies and engineered receptors are impractical to manufacture in large amounts. In addition, they are potentially immunogenic compounds that may elicit antibody responses, potentially limiting the long-term efficacy of such treatments<sup>3,8</sup>. The aim of the current study was to develop a method to enable the immune system of an individual to produce endogenous TNF- $\alpha$  antibodies. This was achieved by immunizing with recombinant murine mTNF- $\alpha$  molecules, modified to contain foreign immunodominant T-helper (T<sub>H</sub>) epitopes. The therapeutic effects of this approach are demonstrated in mouse models of experimental cachexia and collagen-induced arthritis (CIA).

## Results and discussion

**Recombinant T<sub>H</sub> epitope modification of mTNF- $\alpha$ .** A panel of five modified recombinant mTNF- $\alpha$  proteins was produced (Fig. 1D). In each of the constructs, a different segment of mTNF- $\alpha$  was replaced with either a T<sub>H</sub> epitope from ovalbumin OVA(325-333)<sup>9</sup>, which binds to the mouse major histocompatibility complex (MHC) class II molecule H-2A<sup>d</sup>; or with an epitope from hen egg-white lysozyme (HEL), HEL(81-95)<sup>10</sup>, which binds to H-2E<sup>k</sup>. Wild-type and modified mTNF- $\alpha$  proteins were expressed in *Escherichia coli* and purified. The TNF- $\alpha$ -susceptible cell line L929 (ref. 11) was used to assess the biological activity of the molecules. Although concentrations of <20 pg/ml of recombinant wild-type mTNF- $\alpha$  killed the L929 cells, the modified TNF- $\alpha$  molecules were nontoxic in the entire dosage range tested (25-100 pg/ml) (data not shown).

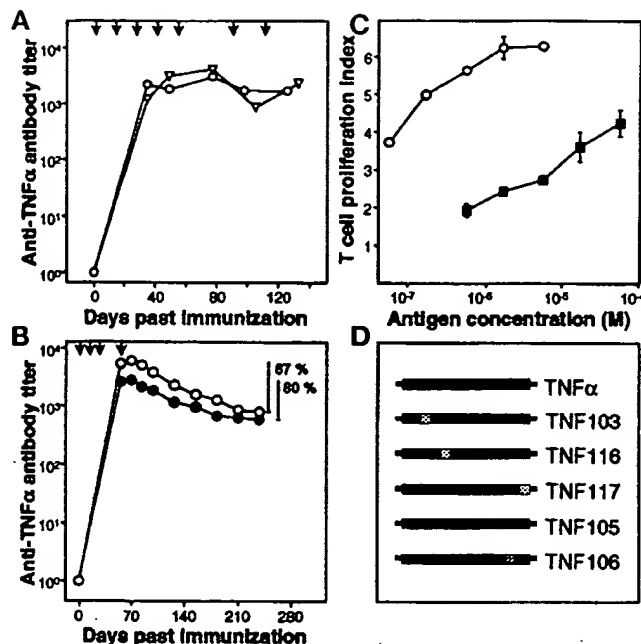
Characterization of antibodies elicited against modified mTNF- $\alpha$ . Groups of mice were immunized with each of the TNF- $\alpha$  molecules, and serum samples were tested for reactivity against nonmodified mTNF- $\alpha$  by ELISA. Mice immunized with the modified TNF- $\alpha$  mol-

ecules TNF103, TNF106, TNF116, and TNF117 rapidly developed high-titer anti-TNF- $\alpha$  antibody responses. This is shown for TNF106 in BALB/c and C3H strains (Fig. 1A, see also Fig. 2A). The TNF105 molecule was poorly immunogenic, but a weak antibody response was detected (data not shown). This demonstrates that insertion of a foreign immunodominant T<sub>H</sub> epitope into mTNF- $\alpha$  can result in a highly immunogenic molecule.

It is widely believed that tolerance within the B-cell population is maintained by clonal deletion and/or by induction of anergy in the population of potentially autoreactive B cells<sup>12,13</sup>. The results shown in Figure 1A, however, demonstrate the existence of a significant population of nonanergic autoreactive B cells, and thus support the notion that lack of T-cell help to these cells is an important mechanism responsible for B-cell tolerance.

High-titer anti-TNF- $\alpha$  antibody levels were maintained by repeating booster injections regularly (Fig. 1A). In a separate experiment in which higher absolute anti-mTNF- $\alpha$  titer values were obtained, it was demonstrated that the titers start to decline one month after the last boost, and after six months the anti-mTNF- $\alpha$  antibody titers have dropped by 80-87% (Fig. 1B). Further supporting the decline of antibody titers to very low levels, we have shown that similarly elicited antibodies against another self protein (ubiquitin) decline by 95% after 22 weeks if the mice are not boosted with the modified antigen (data not shown). The majority of the anti-TNF- $\alpha$  autoantibodies in sera from mice immunized with the modified TNF- $\alpha$  molecules were of the IgG subtype (data not shown) indicating mature T-cell-dependent immune responses. T cells specific for TNF106 as well as for the inserted epitopes could be elicited upon immunization of C3H mice with TNF106 protein (Fig. 1C). Together, these results further support the hypothesis<sup>14</sup> that an effective immune response can be generated against a biologically relevant and disease-associated self protein, TNF- $\alpha$ , by inserting a T<sub>H</sub> epitope into its sequence.

It is conceivable that insertions of T-cell epitopes in different positions in TNF- $\alpha$  (Fig. 1D) create molecules with partly modified tertiary structures compared with the native TNF- $\alpha$  molecule. Therefore, the differently modified proteins could potentially induce



**Figure 1.** Immunization with modified mTNF- $\alpha$  molecules elicits autoantibodies cross-reactive with native mTNF- $\alpha$ . (A) Anti-mTNF- $\alpha$  titers in sera from groups of 20 BALB/c ( $\nabla$ ) or C3H ( $\circ$ ) mice immunized with TNF106. (B) Anti-mTNF- $\alpha$  titers in sera from groups of 45 C3H mice immunized with TNF103 ( $\bullet$ ) or TNF106 ( $\circ$ ). The mice were boosted at the days indicated by arrows. The percentage drops in anti-mTNF- $\alpha$  titers in the two groups are indicated. (C) Proliferation of T cells from C3H mice immunized with TNF106 and stimulated in vitro with serial dilutions of TNF106 ( $\circ$ ) or synthetic HEL(81-96) peptide ( $\blacksquare$ ). (D) Localization in mTNF- $\alpha$  of the foreign T<sub>H</sub> epitopes OVA(325-333) (dotted) and HEL(81-96) (hatched) in TNF103, TNF116, TNF117, TNF105, and TNF106.

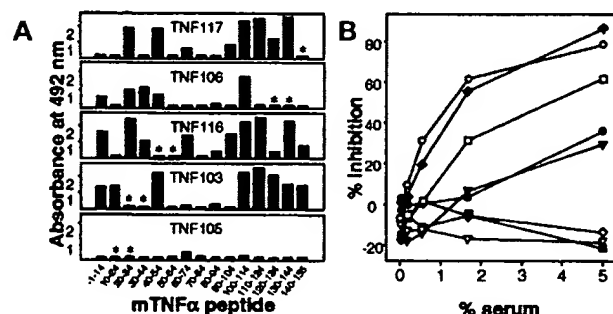
antibodies that are cross-reactive with different parts of the native TNF- $\alpha$  molecule. This was analyzed in several ways. First, the specificities of the polyclonal antisera raised against the modified TNF- $\alpha$  molecules were compared. Overlapping peptides representing the entire mTNF- $\alpha$  sequence were immobilized in microtiter plates, and the antisera were tested for their reactivity with the individual peptides<sup>15</sup>. Although only specificities against linear (presumably nonconformational) B-cell epitopes were detected in this assay, marked qualitative differences in the fine specificities were observed (Fig. 2A), confirming that the intramolecular position of the inserted T-cell epitope influences the specificity of the antibody response.

Second, the ability of antibodies elicited by the modified TNF- $\alpha$  molecules to interfere with the tertiary TNF- $\alpha$  structure was examined in a biochemical assay measuring the binding of mTNF- $\alpha$  to the 55 kDa TNF receptor 1 (TNFR1). Antisera raised against differently modified TNF- $\alpha$  molecules were able to inhibit the receptor-ligand interaction with varying potencies, and with the anti-TNF106 antiserum exhibiting the strongest inhibitory activity (Fig. 2B). By comparing the results in Figures 2A and 2B, it can be postulated that the mTNF- $\alpha$  region comprising amino acids 20-54 contains important neutralizing B-cell epitopes. This is in agreement with published data showing that amino acids within this segment of human TNF- $\alpha$  are important for the receptor interaction<sup>16,17</sup>.

Therapeutic effects of antibodies induced against modified mTNF- $\alpha$ . In order to establish whether the elicited anti-TNF- $\alpha$  antibodies were able to neutralize TNF- $\alpha$  in vivo, a model of experimental cachexia was used. Daily injections of mTNF- $\alpha$  into mice led to severe symptoms such as fever, anorexia, and in many cases, death within 1-5 days. Surviving animals lost up to 20% of their body weight<sup>18</sup>. TNF106

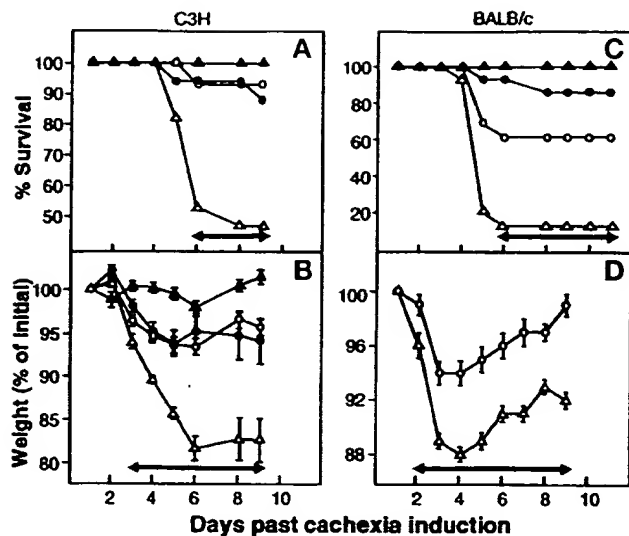
and TNF103, representing a strong and an intermediate elicitor of neutralizing antibodies, respectively, were chosen for these studies. Induction of anti-mTNF- $\alpha$  antibodies preceding the induction of experimental cachexia reduced the mortality of C3H mice from 53% in nonvaccinated mice to 12% in TNF103- vaccinated mice (Fig. 3A). In C3H mice vaccinated with TNF106, the mortality was only 7% (Fig. 3A). In BALB/c mice, the mortality rate was reduced from 87% in nonvaccinated mice to 14% and 38% in TNF103- and TNF106-vaccinated mice, respectively (Fig. 3C). In addition, the weight losses were reduced at least three times in vaccinated mice compared with nonvaccinated controls (Fig. 3B and 3D). The antibodies raised in Balb/c mice against TNF106 have a higher neutralizing capacity in vitro than the antibodies elicited by TNF103 (Fig. 2B). However, this relationship does not exist with regard to inhibition of experimental cachexia in the same mouse strain (Fig. 3C). This suggests that the in vivo effect of the anti-TNF- $\alpha$  antibodies in this model is to bind the administered free TNF- $\alpha$  locally before it can induce the symptoms systemically. The effect thus seems to be more related to the avidity of the induced antibodies rather than the ability to interfere with TNFR1 binding.

We next examined the effects of active immunization against mTNF- $\alpha$  on the enhanced endogenous production of TNF- $\alpha$  in the murine model of rheumatoid arthritis, CIA<sup>19</sup>. DBA/1 mice were immunized four times at two week intervals with either TNF106 or HEL protein as a control. Subsequently, the mice were immunized with bovine collagen type II (CII), and swelling of the joints commenced between 18 and 32 days later. In mice vaccinated with TNF106, the incidence of arthritis was reduced to 60% compared with 90% arthritic mice in the HEL-immunized group (Fig. 4A). These results have therapeutic implications in regard to reduction of relapses in chronic inflammatory diseases. It is important to note that pretreatment with a TNF- $\alpha$ -specific monoclonal antibody had no significant effect on the arthritis incidence in this model<sup>2</sup>. The clinical symptoms of TNF106-vaccinated mice were also ameliorated as shown by significant differences in the clinical score values from day 2 of arthritis onset through to day 10 (Fig. 4B). This improved clinical status is due to a significant reduction in both the level of paw swelling and the number of limbs involved (Fig. 4C and D). In another series of CIA experiments, active immunization with TNF103 also reduced the incidence of disease and severity of clinical symptoms, although to a lesser extent than immunization with TNF106 (data not shown).



**Figure 2.** The anti-mTNF- $\alpha$  fine specificity and neutralizing ability of antisera raised by immunization with modified mTNF- $\alpha$  molecules. (A) Reactivities with overlapping 15- or 16-mer mTNF- $\alpha$  peptides of pooled sera from groups of 5-10 BALB/c mice immunized with TNF117, TNF106, TNF116, TNF103, or TNF105 with boosts at days 14, 28, and 42. The approximate localizations of the foreign T<sub>H</sub> epitopes in the modified TNF- $\alpha$  molecules are indicated by \*. (B) Inhibition of the interaction between mTNF- $\alpha$  and TNFR1 by antisera from mice immunized with TNF117 ( $\blacktriangledown$ ), TNF106 ( $\circ$ ), TNF116 ( $\square$ ), TNF103 ( $\triangle$ ), or TNF105 ( $\blacksquare$ ). Preimmune sera ( $\circ$ ) and sera from BALB/c mice immunized with PBS in adjuvant ( $\nabla$ ) were included as negative controls and a polyclonal rabbit anti-mTNF- $\alpha$  antiserum ( $\blacklozenge$ ) as a positive control.

## RESEARCH



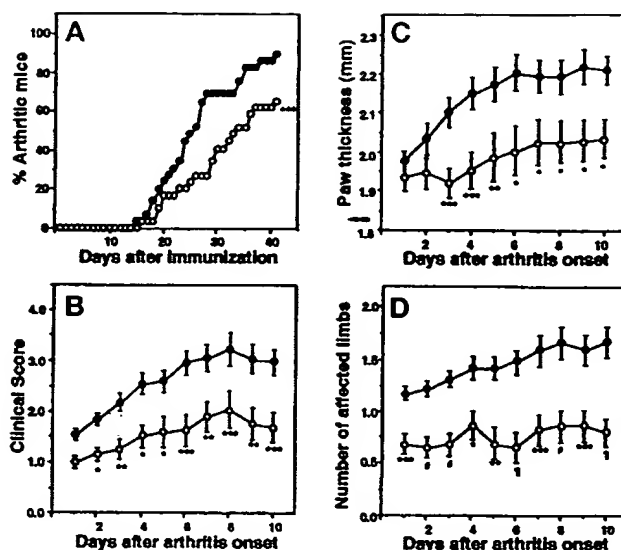
**Figure 3.** Immunization with modified mTNF- $\alpha$  molecules reduces experimental cachexia in mice. Survival rates (A and C) as well as the body weights of surviving mice (B and D) in groups of 14–30 C3H or BALB/c mice immunized with TNF103 (●), TNF106 (○), or PBS (Δ) and two weeks after the last immunization injected intraperitoneally daily with biologically active mTNF- $\alpha$ . A group of control mice (Δ) from each immunization group received PBS intraperitoneally. Error bars represent one standard error of mean. Time intervals with statistically significant differences between vaccinated and control mice are indicated by horizontal arrows ( $p$  values ranged between <0.018 and <0.001).

In the experiments illustrated in Figure 4, the level of reduction of disease symptoms obtained by active immunization against mTNF- $\alpha$  is comparable to that obtained in a previously published study using anti-mTNF- $\alpha$  monoclonal antibodies therapeutically in the same experimental model<sup>2</sup>.

A potential and important concern with vaccination against self proteins is that injection of modified self proteins might induce a permanent autoimmune condition. However, we showed that the anti-TNF- $\alpha$  titers in TNF- $\alpha$ -immune mice declined by 80–87% after termination of the immunizations (Fig. 1B). Whether the residual anti-TNF- $\alpha$  antibodies can still suppress TNF- $\alpha$  remains to be established. The general body weights and mortality rates of TNF- $\alpha$ -immune mice were observed over a period of three months, and no statistically significant differences were found when compared with normal mice (data not shown). No antibody reactivity was detected against the self peptides representing the mTNF- $\alpha$  regions that were replaced with foreign T<sub>H</sub> epitopes (Fig. 2A). Such reactivity could have been elicited if an autoimmune condition driven by endogenously produced TNF- $\alpha$  had occurred (e.g., by epitope spreading<sup>20</sup>). It has been reported that 6% of rheumatoid arthritis patients treated with an anti-TNF- $\alpha$  monoclonal antibody developed treatment-related IgM autoantibodies against double-stranded DNA<sup>8</sup>, a common sign of autoimmune disease. In contrast, antibodies against double-stranded DNA could not be detected in sera from 15 mice actively immunized with modified mTNF- $\alpha$  (data not shown).

Although TNF- $\alpha$  is a mediator of inflammatory disease, it also plays a role in the defense against certain microorganisms. For example, enhanced susceptibility to injected *Listeria monocytogenes* was observed in knockout mice deficient in TNF- $\alpha$  or TNF- $\alpha$  receptors<sup>21–23</sup>. However, rheumatoid arthritis patients treated with monoclonal anti-TNF- $\alpha$  antibodies do not exhibit increased incidence of infections compared with control patients<sup>5</sup>.

The T<sub>H</sub> epitopes inserted into mTNF- $\alpha$  each bind to a known mouse MHC class II allelic type<sup>9,10</sup>. Indeed, it was shown in one



**Figure 4.** Immunization with mTNF- $\alpha$  ameliorates the symptoms of CIA. Groups of 29 DBA/1 mice immunized with TNF106 (○) or HEL (●) were intradermally immunized with bovine CII one week after the last TNF106/HEL immunization. The clinical parameters measured were (A) time of disease onset; (B) clinical score; (C) swelling of the first affected hind limb; (D) the number of arthritic limbs. (B–D) Values are expressed as mean  $\pm$  one standard error of mean.  $\infty$ :  $p < 0.05$ , \*:  $p < 0.02$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.005$ , #:  $p < 0.002$ , †:  $p < 0.0005$ .

experiment that a T<sub>H</sub> response specific for the inserted HEL epitope is induced in C3H mice upon immunization with TNF106 (Fig. 1C). However, it is interesting to note that the modified TNF- $\alpha$  molecules are also immunogenic in inbred strains with nonmatching MHC haplotypes (Fig. 1A and B). This is likely to be due to T cells raised against novel epitopes consisting of inserted foreign epitope sequence and flanking self protein sequence. In a model study, at least two different T<sub>H</sub> cell specificities (against the inserted epitope as well as against a novel flanking epitope) were found to operate during the antibody response toward a self protein modified to contain a foreign T<sub>H</sub> epitope<sup>14</sup>. This observation, combined with the use of promiscuous T<sub>H</sub> epitopes<sup>24</sup>, raises the possibility of using such vaccines in outbred populations such as in humans.

We have provided a novel approach for generating long-term antibody responses against disease-related self proteins such as TNF- $\alpha$  in arthritis. Although anti-TNF- $\alpha$  monoclonal antibodies have proved to be safe and highly effective in these diseases, the treatment is impractical because of the frequent injections and the large quantities of protein required. In this study, we have raised polyclonal antibodies against TNF- $\alpha$  by active immunization with modified TNF- $\alpha$  proteins and shown that this is effective in reducing experimental cachexia as well as the severity and incidence of CIA. Vaccination with modified TNF- $\alpha$  molecules could either be used independently or in combination with monoclonal antibody therapy to prevent future relapses of the disease. The principle of raising antibodies that are cross-reactive with pathogenic self proteins could also be applied to other diseases, such as cancer, allergy, osteoporosis, and atherosclerosis, using the appropriate autologous target proteins.

#### Experimental protocol

**Production of recombinant mTNF- $\alpha$  molecules.** Modifications of the mTNF- $\alpha$  gene were made by introducing oligonucleotides encoding the T<sub>H</sub> epitopes into the DNA sequence using standard PCR mutagenesis procedures. In TNF103, amino acids 24–34 of mTNF- $\alpha$  were replaced with the ovalbumin

sequence QAVHAAHAET; in TNF116, mTNF- $\alpha$  amino acids 49–59 were replaced with the ovalbumin sequence VHAHAHAET; and in TNF117, mTNF- $\alpha$  amino acids 148–157 were replaced with the ovalbumin sequence QAVHAAHAET. In TNF105 and TNF106 mTNF- $\alpha$  amino acids 4–19 and 124–138, respectively, were replaced with HEL sequence SALLSSDITASVNC. In order to minimize the disruption in the overall structures of the modified molecules, mTNF- $\alpha$  sequences with relatively high homology in the primary and predicted secondary structures to the T<sub>H</sub>1 epitopes were exchanged. Wild-type and modified TNF- $\alpha$  molecules were expressed from *E. coli* and purified from solubilized inclusion bodies by ion exchange chromatography to a purity of at least 80% for the modified TNF- $\alpha$  proteins and >95% for wild-type mTNF- $\alpha$  used in ELISA assays.

Immunizations with modified mTNF- $\alpha$  and anti-mTNF- $\alpha$  titer determinations. C3H/He (H-2<sup>a</sup>) and BALB/c (H-2<sup>d</sup>) mice were obtained from M&B (Ry, Denmark), and DBA/1 (H-2<sup>k</sup>) were purchased from Harlan (Bicester, UK). Immunizations with modified mTNF- $\alpha$  were performed by subcutaneous injections of 100  $\mu$ g antigen in phosphate-buffered saline (PBS), emulsified 1:1 in Complete Freund's Adjuvant (CFA; Sigma, St. Louis, MO). Control mice received PBS in CFA. Booster injections were performed similarly using Incomplete Freund's Adjuvant (Sigma). Serum samples were obtained regularly and analyzed in ELISA for reactivity with highly purified recombinant nonmodified mTNF- $\alpha$  (0.1  $\mu$ g/well) immobilized on MaxiSorp (Nunc, Roskilde, Denmark) microtiter plates. The ability of mouse antisera to react with mTNF- $\alpha$  was recorded by absorbance at 492 nm using horseradish peroxidase-labeled rabbit anti-mouse Ig (DAKO, Glostrup, Denmark) as secondary antibody as described<sup>14</sup>. The anti-mTNF- $\alpha$  titer is the dilution obtained when correlating the OD<sub>492</sub> value of a sample to a standard curve (included on each plate) of a high-titer anti-mTNF- $\alpha$  antiserum.

T-cell proliferative assay. C3H/He mice were immunized subcutaneously in the hind footpads and at the tail base with 200  $\mu$ l of a 1:1 emulsion of CFA and 50  $\mu$ g of TNF106 dissolved in PBS. After 10 days, draining lymph nodes were removed, single-cell suspensions prepared, and serial dilutions of antigen mixed with  $2 \times 10^5$  cells/well in microtiter plates (Nunc) in total volumes of 200  $\mu$ l/well of complete RPMI 1640 (Life Technologies, Rockville, MD) containing 1% fresh syngeneic mouse serum. After an 18 h pulse with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine (Amersham, Arlington Heights, IL) per well at the end of a four-day incubation period, the proliferative response was measured on harvested cells in a scintillation counter (Packard Instruments, Meriden, CT). Data were presented as T-cell proliferation indices calculated as the [<sup>3</sup>H]thymidine incorporation of cells cultured with antigen relative to the [<sup>3</sup>H]thymidine incorporation of cells cultured with PBS.

Fine specificity of anti-mTNF- $\alpha$  antisera. Overlapping 15- or 16-mer peptides corresponding to native mTNF- $\alpha$  sequences were synthesized, purified by reverse-phase HPLC, and sequenced as described<sup>14</sup>. Peptides were covalently attached to AquaBind microtiter plates (M&E Biotech, Hørsholm, Denmark), and serial dilutions of pooled antisera were added as described<sup>15</sup>. The reactivities of the antisera were detected as above and compared at 1:200 serum dilution.

Neutralizing ability of anti-mTNF- $\alpha$  antisera. Dilutions of antisera were added to MaxiSorp microtiter plates (Nunc) coated with 50 ng/well human TNFR1 (R&D Systems, Minneapolis, MN). Biotinylated mTNF- $\alpha$  was added (16 ng/well), and bound mTNF- $\alpha$  was subsequently detected by absorbance at 492 nm using streptavidin-peroxidase (DAKO).

Experimental cachexia. C3H/He or BALB/c mice were immunized with modified mTNF- $\alpha$  as described already. Two weeks after the last immunization, an appropriate dose (10–40  $\mu$ g) of biologically active mTNF- $\alpha$  was injected intraperitoneally daily in all mice except control mice, which received PBS administered intraperitoneally. The survival rates and the body weights of surviving mice were determined. The experiments were repeated nine times with similar results. Statistical analyses of the differences between vaccinated and control mice were performed using Mann-Whitney's rank sum test (survival rates) or Fisher's exact test (relative body weights).

Collagen-induced arthritis. Five-week-old male DBA/1 mice (CIA-susceptible mouse strain, H-2<sup>k</sup>) were immunized as described already with TNF106 or HEL with three subsequent booster injections at days 14, 28, and 42. At 12 weeks of age, the mice were immunized intradermally at the base of the tail with 200  $\mu$ g CII prepared as described<sup>25</sup> and emulsified 1:1 in CFA. From day 15 after CII immunization, the mice were examined daily for onset of CIA using two clinical parameters: paw swelling and clinical score<sup>2</sup>. Paw swelling was assessed by measuring the thickness of the affected hind paws with 0–10 mm calipers (Kroepelin, Schluchtern, Germany). Values for the clinical score were assigned as follows: 0 = normal; 1 = slight swelling and

erythema; 2 = pronounced edema; and 3 = joint rigidity. Each limb was graded, resulting in a maximal clinical score of 12 per animal. The arthritis was monitored over 10 days, after which the mice were killed. Nonarthritic mice were graded as clinical score = 0; number of affected limbs = 0; and paw thickness = 1.8 mm. The t-test was used to compare the onset of arthritis between treatment groups. The Mann-Whitney test to compare nonparametric data for statistical significance was applied the other clinical data.

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1. Thorbecke, G.J. *et al.* Involvement of endogenous tumor necrosis factor alpha and transforming growth factor beta during induction of collagen type II arthritis in mice. *Proc. Natl. Acad. Sci. USA* **89**, 7375–7379 (1992).
2. Williams, R.O., Feldmann, M. & Maini, R.N. Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. *Proc. Natl. Acad. Sci. USA* **89**, 9784–9788 (1992).
3. Williams, R.O., Ghayeb, J., Feldmann, M. & Maini, R.N. Successful therapy of collagen-induced arthritis with TNF receptor-IgG fusion protein and combination with anti-CD4. *Immunology* **84**, 433–439 (1995).
4. Neurath, M.F. *et al.* Predominant pathogenic role of tumor necrosis factor in experimental colitis in mice. *Eur. J. Immunol.* **27**, 1743–1750 (1997).
5. Elliott, M.J. *et al.* Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor alpha. *Arthritis Rheum.* **36**, 1681–1690 (1993).
6. Moreland, L.W. *et al.* Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein. *N. Engl. J. Med.* **337**, 141–147 (1997).
7. Targan, S.R. *et al.* A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor-alpha for Crohn's disease. *Crohn's Disease cA2 Study Group. N. Engl. J. Med.* **337**, 1029–1035 (1997).
8. Elliott, M.J. *et al.* Repeated therapy with monoclonal antibody to tumour necrosis factor alpha (cA2) in patients with rheumatoid arthritis. *Lancet* **344**, 1125–1127 (1994).
9. Sette, A. *et al.* Structural characteristics of an antigen required for its interaction with Ia and recognition by T cells. *Nature* **328**, 395–399 (1987).
10. Shastri, N., Gammon, G., Horvath, S., Miller, A. & Sercarz, E.E. The choice between two distinct T cell determinants within a 23-amino acid region of lysozyme depends on their structural context. *J. Immunol.* **137**, 911–915 (1986).
11. Ruff, M.R. & Gifford, G.E. Purification and physico-chemical characterization of rabbit tumor necrosis factor. *J. Immunol.* **125**, 1671–1677 (1980).
12. Nemazee, D.A. & Burki, K. Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. *Nature* **337**, 562–566 (1989).
13. Goodnow, C.C. *et al.* Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature* **334**, 676–682 (1988).
14. Dalum, I., Jensen, M.R., Hindersson, P., Elsner, H.I. & Mouritsen, S. Breaking of B cell tolerance toward a highly conserved self protein. *J. Immunol.* **157**, 4796–4804 (1996).
15. Dalum, I. *et al.* Induction of cross-reactive antibodies against a self protein by immunization with a modified self protein containing a foreign T helper epitope. *Mol. Immunol.* **34**, 1113–1120 (1997).
16. Eck, M.J. & Sprang, S.R. The structure of tumor necrosis factor-alpha at 2.6 Å resolution. Implications for receptor binding. *J. Biol. Chem.* **264**, 17595–17605 (1989).
17. Bloom, J.W., Bettencourt, J.D. & Mitra, G. Epitope mapping and functional analysis of three murine IgG1 monoclonal antibodies to human tumor necrosis factor- $\alpha$ . *J. Immunol.* **151**, 2707–2716 (1993).
18. Cerami, A., Ikeda, Y., Le Trang, N., Hotez, P.J. & Beutler, B. Weight loss associated with an endotoxin-induced mediator from peritoneal macrophages: the role of cachectin (tumor necrosis factor). *Immunol. Lett.* **11**, 173–177 (1985).
19. Staines, N.A. & Wooley, P.H. Collagen arthritis—what can it teach us? *Br. J. Rheumatol.* **33**, 798–807 (1994).
20. Lehmann, P.V., Forsthuber, T., Miller, A. & Sercarz, E.E. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* **358**, 155–157 (1992).
21. Pasparakis, M., Alexopoulou, L., Episkopou, V. & Kollias, G. Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. *J. Exp. Med.* **184**, 1397–1411 (1996).
22. Rothe, J. *et al.* Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by *Listeria monocytogenes*. *Nature* **364**, 798–802 (1993).
23. Erickson, S.L. *et al.* Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. *Nature* **372**, 560–563 (1994).
24. Panina-Bordignon, P. *et al.* Universally immunogenic T cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T cells. *Eur. J. Immunol.* **19**, 2237–2242 (1989).
25. Williams, R.O., Williams, D.G. & Maini, R.N. Anti-type II collagen ELISA. Increased disease specificity following removal of anionic contaminants from salt-fractionated type II collagen. *J. Immunol. Methods* **147**, 93–100 (1992).

# Active Vaccination Against IL-5 Bypasses Immunological Tolerance and Ameliorates Experimental Asthma<sup>1</sup>

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Current therapeutic approaches to asthma have had limited impact on the clinical management and resolution of this disorder. By using a novel vaccine strategy targeting the inflammatory cytokine IL-5, we have ameliorated hallmark features of asthma in mouse models. Delivery of a DNA vaccine encoding murine IL-5 modified to contain a promiscuous foreign Th epitope bypasses B cell tolerance to IL-5 and induces neutralizing polyclonal anti-IL-5 Abs. Active vaccination against IL-5 reduces airways inflammation and prevents the development of eosinophilia, both hallmark features of asthma in animal models and humans. The reduced numbers of inflammatory T cells and eosinophils in the lung also result in a marked reduction of Th2 cytokine levels. Th-modified IL-5 DNA vaccination reduces the expression of IL-5 and IL-4 by ~50% in the airways of allergen-challenged mice. Most importantly, Th-modified IL-5 DNA vaccination restores normal bronchial hyperresponsiveness to  $\beta$ -methacholine. Active vaccination against IL-5 reduces key pathological events associated with asthma, such as Th2 cytokine production, airways inflammation, and hyperresponsiveness, and thus represents a novel therapeutic approach for the treatment of asthma and other allergic conditions. *The Journal of Immunology*, 2001, 167: 3792–33799.

Interleukin-5 is a proinflammatory cytokine expressed at high levels in asthmatics. Asthma is clinically characterized by episodic airflow obstruction, inflammation of the airways, and enhanced bronchial reactivity to nonspecific spasmogens. The levels of airways obstruction and hyperreactivity (AHR)<sup>4</sup> often correlate with the degree of airways inflammation, and these clinical features are indicative of asthma severity (1–7). Clinical correlates between the degree of cellular infiltration and disease progression have identified inflammation of the airways as the major contributing factor to pathogenesis and pathobiology. The inflammatory infiltrate in asthma is complex; however, it is now widely recognized that CD4<sup>+</sup> Th lymphocytes with a Th2 profile (Th2 cells) of cytokine expression play a pivotal role in the clinical expression and pathogenesis of this disorder (8, 9). Th2 cells regulate disease progression and AHR by orchestrating allergic inflammation of the airways through the release of a range of cytokines (IL-4, -5, -9, -10, -13) (10–13). Like Th2 cells, the levels of eosinophils and their inflammatory products in the lung correlate

with disease severity, and accumulation of this leukocyte in the airways is a central feature of bronchial dysfunction during the late-phase asthmatic response (14). Although Th2 cells orchestrate many facets of the allergic response, their role in regulating eosinophilia through the secretion of IL-5 is thought to be a major proinflammatory pathway in asthma.

The central role of IL-5 in regulating eosinophil function (differentiation, expansion, mobilization, and activation) has identified this cytokine as a primary target for therapeutic intervention in asthma. Indeed, the importance of IL-5 in regulating eosinophilia and potentially asthma pathogenesis has been demonstrated in experimental systems that have used animal models of asthma in conjunction with IL-5-deficient mice and inhibitory mAbs (15–17). These studies have highlighted the need to develop advanced methodologies that target IL-5 function for the resolution of both inflammation and AHR in asthma.

The aim of the current investigation was to induce a therapeutic immune response directed against self-IL-5. Although anti-IL-5 mAbs are being used in clinical trials in Ag challenge studies, this cytokine has never been used as a target for active vaccination. Here we describe a novel therapeutic DNA vaccine approach for the treatment of allergy and asthma. Our strategy was to use active DNA vaccination against IL-5 to elicit polyclonal Abs that would neutralize IL-5 produced during recall responses to inhaled allergen and ameliorate disease. This therapeutic approach has the potential advantage not only of providing protection during asthma exacerbation, but also as a longitudinal anti-inflammatory therapy. We have shown previously that by incorporating a strong Th cell epitope within a self-protein, immunological tolerance against self-proteins can be bypassed (18–20). In the current investigation, we extended these studies by using a DNA vaccine encoding murine IL-5 modified to contain a promiscuous foreign Th epitope to break or bypass immunological tolerance to IL-5. In mice, Th-modified IL-5 DNA vaccination induced an immune response directed against native IL-5. Moreover, in our established models of experimental asthma, Th-modified IL-5-vaccinated mice induced an immune response directed against IL-5 that reduced both

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<sup>4</sup> Abbreviations used in this paper: AHR, airways hyperreactivity; BALF, bronchoalveolar lavage fluid; HPRT, hypoxanthine-guanine phosphoribosyltransferase; mIL, mouse IL; Penh, enhanced pause; wt, wild type; PBLN, peribronchial lymph node.

pulmonary lymphocyte infiltration and eosinophilia, reduced the levels of Th2 cytokines, and inhibited the development of AHR. These data substantiate active DNA vaccination against IL-5 as a novel therapeutic approach for the treatment of asthma and potentially other allergic diseases. In addition, our vaccine approach is a platform technology that can be applied to most self-proteins that are aberrantly or overexpressed during chronic disease.

## Materials and Methods

### Mice

Male C3H/HeN (H-2<sup>b</sup>) or C3H/HeJ (H-2<sup>k</sup>) mice (6–8 wk old) were obtained from M&B A/S (Ry, Denmark) or the specific-pathogen free facility at the John Curtin School of Medical Research, Australian National University. Experiments were performed according to the Danish Animal Experiments Inspectorate and John Curtin School of Medical Research institutional guidelines for animal care and use.

### Modified IL-5 plasmid construction and DNA preparation

Murine IL-5 cDNA (R&D Systems, Minneapolis, MN) was cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) mammalian expression vector containing the mIL-5 signal peptide and a consensus Kozak sequence (21). The P30 tetanus toxoid (22) helper epitope (FNNFTVSFVLRVPKVSASHLE) was inserted into the first loop region of mIL-5, replacing aa R30 to L41, using sequence overlap extension PCR (sense primer, CTTCTGGCTGCGCGTGCCCAAGGTGAGCGCCAGCCACCTGGAGTGCATTGGA GAGATCTTTTCAGGGGC; antisense primer, GCGCTCACCTTGGGCA CGCGCAGCCAGAAGCTCACGGTGAAGTTGTTGAACATCGTCTCA TTGCTTGTCACAGAGC) and standard molecular biology techniques. Plasmid DNA was prepared using endotoxin-free purification kits (Qiagen, Valencia, CA) and was resuspended in sterile 0.9% saline.

### DNA vaccination

Mice were anesthetized, and the lower back of each animal was shaved. Vaccination with mIL-5.wt (encodes the wild-type mouse IL-5 cDNA) or mIL-5.2 (encodes the mIL-5.2 Th-modified construct) was then performed by intradermal injections of 100  $\mu$ l of DNA solution (1  $\mu$ g/ $\mu$ l) into each mouse divided into two injections over the lower back. DNA injections were performed six or seven times at 2-wk intervals. At the end of the sixth vaccination, mice were sensitized to OVA to prime for subsequent induction of allergic airway inflammation (described below). Vaccinations were continued (one injection of DNA every 2 wk) during the period of induction of allergic disease of the lung.

### ELISA

Anti-mIL-5 titers in sera were determined by direct ELISA. Briefly, pooled sera from vaccinated mice were titrated into 96-well flat-bottom plates (Maxisorp; Nunc, Taastrup, Denmark) precoated with mIL-5 (100 ng/well; R&D Systems). Sera were detected with goat anti-mouse-HRP polyclonal Ab (DAKO, Glostrup, Denmark). Competition ELISAs were performed by adding diluted antisera preincubated with mIL-5 for 1 h to 96-well plates coated with anti-mIL-5 mAb (TRFK5.1  $\mu$ g/well; R&D Systems). Inhibition by the antisera was detected by adding biotinylated TRFK4 (R&D Systems) and streptavidin-HRP (Amersham, Hørsholm, Denmark). Serum OVA-specific IgE was detected by isotype-specific ELISA using rat anti-mouse IgE mAb, clone LO-ME-3 (BioSource International, Camarillo, CA). OVA-specific IgE was quantitated against standard mouse IgE.

### Induction of allergic airway inflammation

**Intranasal model.** Mice were sensitized by s.c. injection of 50  $\mu$ g of OVA in 0.9% sterile saline mixed 1/1 (v/v) with Adju-Phos (Superfos Biosector, Vedbaek, Denmark) weekly over 3 wk. Four days after the last sensitization the mice were challenged intranasally with 12.5  $\mu$ g of OVA in 0.9% sterile saline once a day, every other day, for a total of three challenges. Bronchoalveolar lavage fluid (BALF) was collected 1 day following the last challenge.

**Aeroallergen models: sensitization regimen.** Mice were sensitized by i.p. injection with 50  $\mu$ g of OVA/1 mg of Alhydrogel (CSL, Parkville, Australia) in 0.9% sterile saline. Nonsensitized mice received 1 mg of Alhydrogel in 0.9% saline. On days 12, 14, 16, and 18, all groups of mice were aeroallergen challenged with OVA as previously described (15, 23). Blood was collected on days 13, 15, 17, and 19. Twenty-four hours after the last aeroallergen challenge AHR was measured, and then BALF and lung tissue were collected for analysis of inflammatory infiltrates.

### Generation and transfer of Th2 cells and induction of allergic disease of the lung

T cell donor mice (male C3H/HeJ, 6–8 wk of age) were sensitized by i.p. injection of 50  $\mu$ g of OVA/1 mg of Alhydrogel in 0.9% sterile saline to prime for CD4<sup>+</sup> Th2-like cells. Six days following sensitization donor mice were sacrificed, and their spleens were excised. Splenocytes were then disaggregated, contaminating RBC were lysed, and subsequently washed splenocytes were resuspended at  $5 \times 10^6$  cells/ml in RPMI 1640 culture medium. These cells were then cultured for 4 days at 37°C in the presence of 200  $\mu$ g/ml OVA, 20 ng/ml murine IL-4, and 40  $\mu$ g/ml anti-IFN- $\gamma$  (R46A2). CD4<sup>+</sup> T cells were isolated from cultures using high gradient magnetic MiniMACS separation column (MACS separation) as described previously (23), washed, and resuspended in PBS. CD4<sup>+</sup> T cells ( $2 \times 10^6$  cells) were adoptively transferred to DNA-vaccinated or unvaccinated naive C3H/HeJ mice or were stimulated in vitro with mitomycin-treated APCs in the presence of OVA to determine Th2 cytokine profiles. Twelve and 36 h later, recipients were exposed to an aerosol of OVA (10 mg/ml) in 0.9% saline twice for 30 min each time (30-min interval between exposures). AHR to  $\beta$ -methacholine was determined, and blood, BALF, and lung tissue were collected for the analysis of inflammatory infiltrates 24 h after the last aerosol.

### Characterization of lung morphology and leukocytes in blood, tissue, and BALF

Lung tissue representing the central (bronchi-bronchiole) and peripheral (alveoli) airways was fixed in 10% phosphate-buffered Formalin, sectioned, and stained with Alcian blue-periodic acid-Schiff for the enumeration of mucin-secreting cells or Carbol's chromotrope-hematoxylin for the identification of eosinophils. Eosinophils in blood, BALF, and lung were identified by morphological criteria and quantified as previously described (15, 23).

### Measurement of AHR

Responsiveness to  $\beta$ -methacholine was assessed in conscious unrestrained mice by barometric plethysmography using apparatus and software supplied by Buxco (Troy, NY). This system yields a dimensionless parameter known as enhanced pause (Penh), reflecting changes in waveform of the pressure signal from the plethysmography chamber combined with a timing comparison of early and late expirations. Measurement of Penh was performed essentially as previously described (24, 25). Briefly, mice were placed in the plethysmograph chamber and exposed to an aerosol of water (baseline readings) and then to cumulative concentrations of  $\beta$ -methacholine ranging from 3 to 50 mg/ml. The aerosol was generated by an ultrasonic nebulizer and drawn through the chamber for 2 min. The inlet was then closed, and Penh readings were taken for 3 min and averaged. Values were reported as the percent increase over baseline.

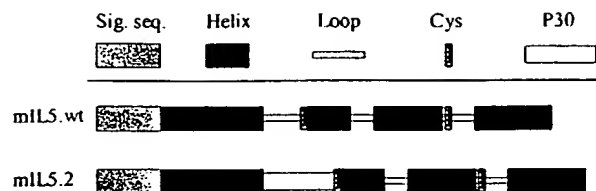
### Measurement of cytokine production by peribronchial lymph nodes

Cells from the peribronchial lymph nodes were isolated and stimulated with 1 mg/ml OVA in MLC medium for 72 h as described previously (25). The concentrations of IL-4, IL-5, and IL-13 in the cell-free supernatants were measured with ELISA (25). The sensitivity of detection was 0.5 ng/ml for IL-5 and IL-13 and 0.1 ng/ml for IL-4.

### RT-PCR analysis

Total RNA was isolated from lungs by standard methods with RNAzol B (Biotecx Laboratories, Houston, TX). A RT-PCR procedure was performed as previously described (26) to determine relative quantities of mRNA for various cytokines. The primers and probes for all genes were purchased from Life Technologies (Gaithersburg, MD). Primer and probe sequences for hypoxanthine-guanine phosphoribosyl transferase (HPRT) have been described previously (26). Primer and probe sequences for IL-4, IL-5, IL-10, IL-13, IFN- $\gamma$ , and HPRT are as follows: IL-4: sense, GAATGT ACCAGGAGCCATATC; antisense, CTCAGTACTACGAGTAATCCA; probe, AGGGCTTCCAAGGTGCTTCGCA; IL-5: sense, GACAAGCA ATGAGACACGATGAGG; antisense, GAACCTGACGAGTAATCCA GG; probe, GGGGGTACTGTGGAAATGCTTAT; IL-10: sense, CGG GAAGACAATAACTG; antisense, CATTCCGATAAGGCTTGG; probe, GGACTGCCCTTCAGCCAGGTGAAGACTTT; IL-13: sense, CTC CCTCTGACCCTTAAGGAG; antisense, GAAGGGCCGTGGCGA AACAG; probe, TCCAATTGCAATGCCATCTAC; and IFN- $\gamma$ : sense, AACGCTACACACTGCATCTTGG; antisense, GACTTCAAAGAGTCT GAGG; probe, GGAGGAAGTGGCAAAGGA. The cycle numbers used for amplification of each gene product are: IL-10, 27 cycles; IL-13 and





**FIGURE 1.** Murine Th-modified IL-5 DNA construct design. Murine IL-5 cDNA was cloned into pcDNA3.1 mammalian expression vector (Invitrogen, San Diego, CA) containing the mIL-5 signal peptide. In mIL-5.2, the P30 promiscuous Th epitope (22) was inserted into the first loop region of IL-5.

IFN- $\gamma$ , 30 cycles; IL-4 and IL-5, 28 cycles; and HPRT, 23 cycles. After the appropriate number of PCR cycles, the amplified DNA was analyzed by gel electrophoresis and Southern blotting and was detected using the ECL detection system as recommended by the manufacturer (Amersham, Arlington Heights, IL). PCR amplification with the HPRT reference gene was performed to assess variations in cDNA or total RNA loading between samples. Mean relative transcript levels per group were determined from cDNA panels as previously described (27). Briefly, values were derived by dividing the mean of the triplicate values measured for the transcript of interest by the mean of triplicate HPRT values for the sample.

#### Statistical analysis

The significance of differences between experimental groups was analyzed using Student's unpaired *t* test. Values were reported as the mean  $\pm$  SEM. Differences in means were considered significant at  $p < 0.05$ .

## Results

### Murine Th-modified IL-5 DNA vaccines can bypass B cell tolerance

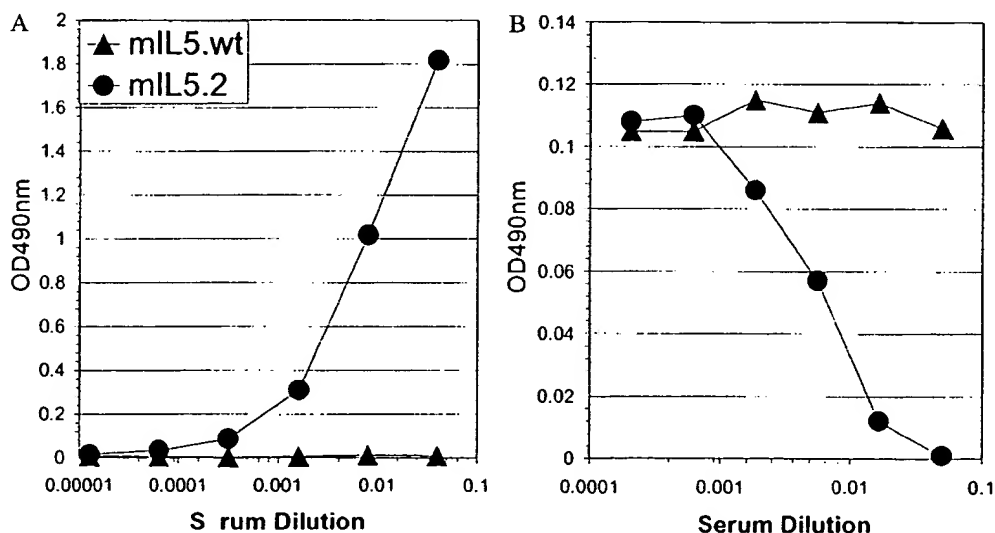
A DNA vaccine encoding murine IL-5 modified to contain a promiscuous foreign Th epitope, P30 from the tetanus toxoid (22) (Th-modified mIL-5.2), was designed and tested for its ability to induce a polyclonal Ab response that was cross-reactive with nonmodified murine IL-5 (mIL-5.wt; see Fig. 1). Before vaccination, the constructs were tested in COS cell transient transfections to ensure that the encoded proteins were appropriately expressed. The

nonmodified wild-type murine IL-5 (mIL-5.wt) and the murine Th-modified IL-5 (mIL-5.2) constructs were both capable of being transiently expressed, as detected by Western blotting (data not shown).

The Th-modified IL-5 construct capable of expressing protein in COS cell transient transfection was further tested for immunogenicity by DNA vaccinations in mice. C3H mice were initially immunized intradermally with 100  $\mu$ g of DNA in 0.9% saline and subsequently boosted (100  $\mu$ g) six times at 2-wk intervals. Although the mIL-5.wt construct was unable to induce an anti-mIL-5 humoral immune response (0 of 40 mice tested), the mIL-5.2 Th-modified construct bypassed B cell immune tolerance and induced polyclonal Abs that were cross-reactive with native murine IL-5 (30 of 30 mice tested; Fig. 2A). Anti-mIL-5 (and anti-P30) Abs could be detected after the third immunization, and 100% of the mice had seroconverted after the fourth mIL-5.2 DNA immunization (data not shown). Antisera from mice vaccinated with mIL-5.2 DNA were tested for its ability to compete for the binding of native murine IL-5 by using a competition ELISA (Fig. 2B). Antisera from vaccinated mice were preincubated with mIL-5 and were capable of competing with an anti-mIL-5 mAb (TRFK4) for the binding of mIL-5 protein. Thus, polyclonal Abs from mIL-5.2-vaccinated mice efficiently blocked the interaction of neutralizing anti-murine IL-5 mAbs TRFK4/TRFK5 with the native ligand. Together these data suggest that the polyclonal Abs induced via mIL-5.2 DNA vaccination recognize native murine IL-5.

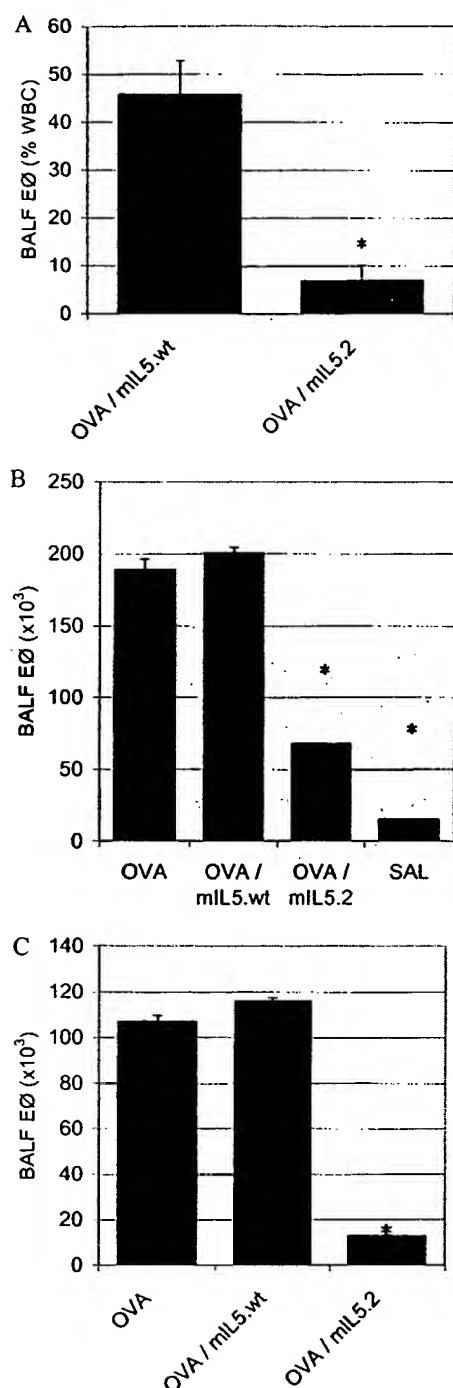
### Eosinophilia and pulmonary lymphocyte inflammation are inhibited in mice vaccinated with Th-modified IL-5 DNA

The mIL-5.2 DNA vaccine was further characterized in three separate mouse models of allergic airways inflammation that mimic key pathological events characteristic of asthma (15, 28). The first model was a simple intranasal OVA-allergen model (intranasal model) that induces eosinophilia in the lungs of mice. This model allowed us to rapidly assess and validate the concept that Th-modified mIL-5.2 DNA vaccination could induce Abs that cross-react with native mIL-5 and reduce eosinophilia in the BALF of a large number of allergic mice. An OVA aeroallergen sensitization model



**FIGURE 2.** Th-modified IL-5 DNA vaccination induces polyclonal Abs that cross-react with native mIL-5. *A*, Anti-mIL-5 serum ELISA with pooled sera from mice vaccinated and boosted five times with 100  $\mu$ g of endotoxin-free mIL-5.wt ( $\blacktriangle$ ) or mIL-5.2 Th-modified ( $\bullet$ ) DNA in 0.9% sterile saline. Abs that cross-react with native murine IL-5 can be detected after the third DNA vaccination, and by the fourth DNA vaccination 100% of the mice have seroconverted. *B*, Competition ELISA of TRFK4/5 (R&D Systems) anti-mIL-5 mAbs and pooled sera from mIL-5.wt ( $\blacktriangle$ ) and mIL-5.2 ( $\bullet$ ) DNA vaccinated mice preincubated with mIL-5.





**FIGURE 3.** BALF eosinophilia is reduced in Th-modified IL-5 DNA vaccinated mice. *A*, OVA intranasal regimen; *B*, sensitization regimen; *C*, T cell transfer/OVA aerosol regimen. The frequency (*A*; mean  $\pm$  SEM of 19 (mIL-5.wt) or 30 (mIL-5.2) mice) and total number (*B* and *C*; mean  $\pm$  SEM of six mice) of eosinophils recovered from BALF of the airway lumen was determined 24 h after the last aeroallergen challenge. \*,  $p < 0.000001$  (*A*),  $p < 0.05$  (*B*), and  $p < 0.005$  (*C*) for OVA-sensitized mIL-5.wt-vaccinated mice compared with OVA-sensitized mIL-5.2-vaccinated mice.

(OVA sensitization model) was then later used to look at the AHR response in vaccinated mice. Thirdly, an adoptive transfer model (transfer model) of allergen-specific Th2 CD4<sup>+</sup> T cells was used to address the ability of the vaccines to reduce disease symptoms in a model in which sensitization to the aeroallergen was performed in an environment free of the effects of the anti-mIL-5 immune

response. The number and frequency of eosinophils in BALF, blood, and lung tissue were assessed in all three models of OVA-induced allergic airways inflammation. In the intranasal model, mice were vaccinated six times with DNA and sensitized to OVA s.c. four times at weekly intervals. One week after the last sensitization, the mice were challenged with OVA intranasally three times over a 6-day period. One day later, BALF was collected for eosinophil counts. As shown in Fig. 3*A*, eosinophilia in BALF was dramatically reduced in mice vaccinated with Th-modified mIL-5.2 ( $n = 30$  mice) compared with mIL-5.wt ( $n = 19$  mice). Next, the effect of mIL-5.2 DNA vaccination on eosinophilia and the subsequent induction of AHR was determined in the OVA sensitization and transfer models of experimental asthma that employed OVA sensitization or adoptive transfer of Ag-specific CD4<sup>+</sup> T cells (that secrete Th2 cytokines such as IL-5 and IL-4) to naive mice before allergen provocation of the lung. In the CD4<sup>+</sup> T cell adoptive transfer model, the Th2 T cells are sensitized to allergen (OVA) in an environment free of neutralizing mIL-5 Abs, thus ruling out a failure to induce the model due to the vaccinations. Eosinophil recruitment into the BALF was inhibited in both aeroallergen models in response to inhaled allergen after mIL-5.2 DNA vaccination (Fig. 3, *B* and *C*, and Table I). Notably, blood eosinophilia was completely attenuated after mIL-5.2 DNA vaccination (Fig. 4, *A* and *B*). Moreover, active DNA vaccination with mIL-5.2 in the transfer model ameliorated tissue eosinophilia in the lungs of all mice (Fig. 4*C*). The inability of mIL-5.2 DNA-vaccinated mice to mount blood eosinophilia directly correlated with the inability of the bone marrow pool of eosinophils to expand in response to allergen provocation of the lung (results not shown). Lymphocyte numbers recruited to the lungs in the OVA sensitization model were also reduced in mIL-5.2 DNA-vaccinated mice. On day 19 after aerosol challenge, there were significantly reduced lymphocyte numbers in the BALF of mIL-5.2 DNA-vaccinated mice compared with wild-type mIL-5 DNA-vaccinated or nonvaccinated controls (Table I). Thus, neutralizing IL-5 via DNA vaccination effectively inhibits the recruitment of inflammatory cells to the sites of allergic inflammation by acting systemically.

#### Th-modified IL-5 DNA vaccination restores normal airways reactivity in OVA-sensitized mice

To determine the ability of mIL-5.2 DNA vaccination to reduce AHR to cholinergic stimuli, lung reactivity to  $\beta$ -methacholine was measured in the OVA sensitization and T cell transfer models 24 h after the last aeroallergen challenge (Fig. 5). Mice vaccinated

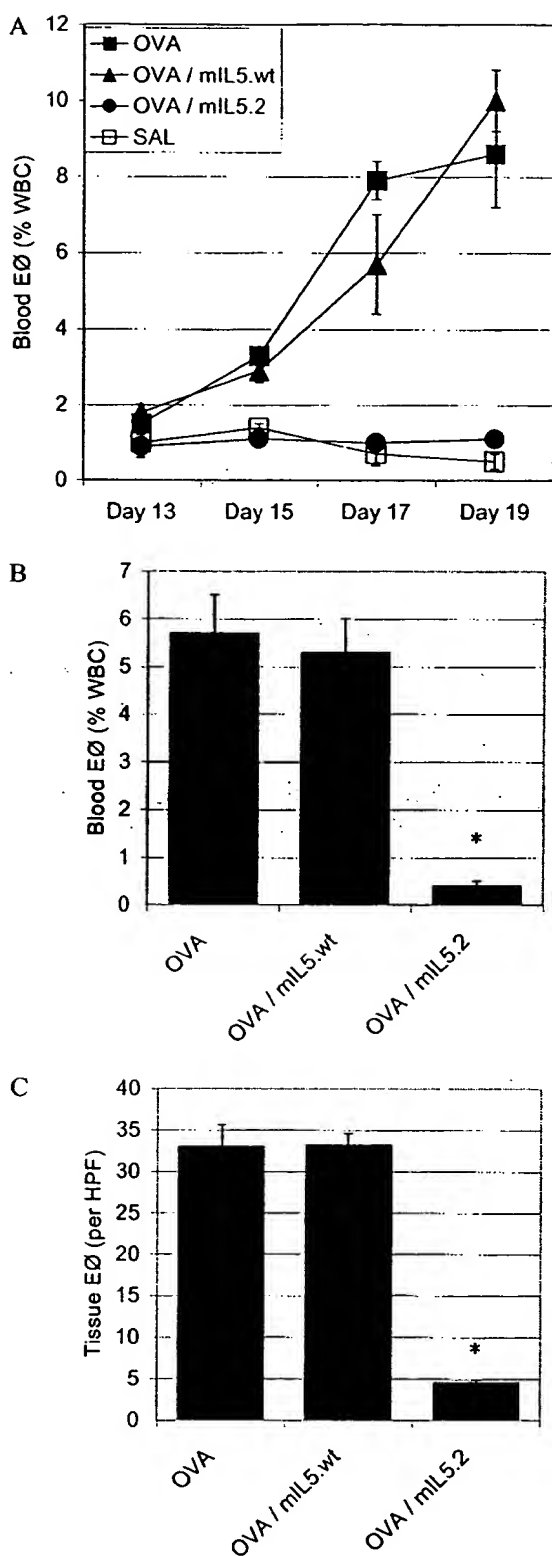
**Table I.** Reduced numbers of inflammatory T cells and eosinophils in the BALF of allergen-challenged mice after Th-modified IL-5 DNA vaccination

	Day 19 BALF <sup>a</sup>	
	T lymphocytes (cells/ml $\times 10^3$ )	Eosinophils (cells/ml $\times 10^3$ )
Saline	19 $\pm$ 12 <sup>b</sup>	15 $\pm$ 6
OVA	221 $\pm$ 43	189 $\pm$ 27
OVA/mIL-5.wt	230 $\pm$ 37	201 $\pm$ 35
OVA/mIL-5.2	110 $\pm$ 26*	68 $\pm$ 19**

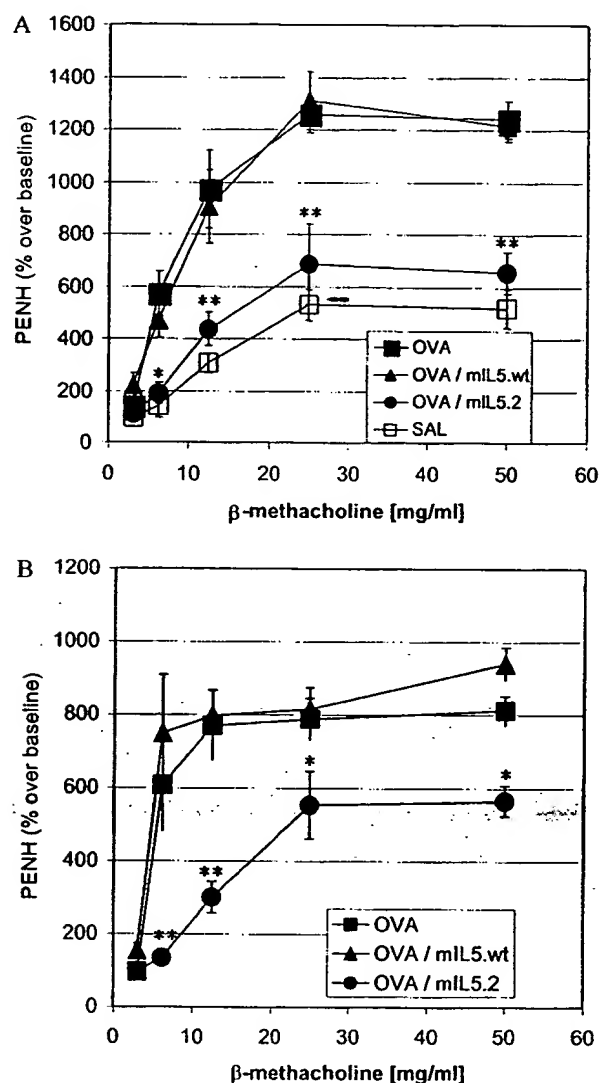
<sup>a</sup> BALF was collected on day 19, one day after the final OVA-aeroallergen challenge as described in *Materials and Methods*, and infiltrating cells were identified morphologically.

<sup>b</sup> Mean cell numbers are shown ( $\pm$  SEM).

\* T lymphocytes  $p < 0.005$  and \*\*, eosinophils  $p < 0.05$  for OVA-sensitized mIL-5.2-vaccinated mice when compared to OVA-sensitized mIL-5.wt-vaccinated mice.



**FIGURE 4.** Blood and lung tissue eosinophilia are reduced in Th-modified IL-5 DNA-vaccinated mice. *A*, The percentages of eosinophils in the peripheral blood on various days using the sensitization regimen. *B*, The percentages of eosinophils in the peripheral blood 24 h after the last aeroallergen challenge in the T cell transfer/OVA aerosol regimen. *C*, The mean number of lung peribronchial/perivascular eosinophils per 10 similar high-powered fields (HPF;  $\times 1000$  magnification) for each group 24 h after the last aeroallergen challenge in the Th2 cells transfer/OVA aerosol regimen. \*,  $p < 0.005$  for OVA-sensitized mL5.wt vaccinated mice compared with OVA-sensitized mL5.2-vaccinated mice (mean  $\pm$  SEM of six mice).



**FIGURE 5.** Reduced AHR to cholinergic stimuli in Th-modified IL-5 DNA-vaccinated mice. *A*, Sensitization regimen; *B*, CD4<sup>+</sup> T cell transfer/OVA aerosol regimen. Reactivity to  $\beta$ -methacholine was measured by barometric plethysmography, and the data (mean of seven mice  $\pm$  SEM) represent the percent increase in Penh over baseline reactivity in the absence of cholinergic stimuli. Heightened reactivity was seen at all concentrations of methacholine by OVA-sensitized mL5.wt-vaccinated mice compared with that in OVA-sensitized mL5.2-vaccinated mice (\*,  $p < 0.05$ ; \*\*,  $p < 0.005$ ).

with mL5.wt DNA developed AHR similar to nonvaccinated OVA-sensitized controls (Fig. 5A) and naive mice that received CD4<sup>+</sup> T cells (Fig. 5B) after aeroallergen challenge. By contrast, in the OVA sensitization model AHR in mice vaccinated with the mL5.2 DNA was reduced to a level similar to that in nonsensitized saline-treated control mice (Fig. 5A). Importantly, we also demonstrated that mL5.2 DNA was effective at inhibiting T cell-regulated AHR. The level of airways reactivity to  $\beta$ -methacholine in the T cell transfer model after mL5.2 DNA vaccination (Fig. 5B) was similar to that observed in saline-treated controls (Fig. 5A). Although there is conflicting evidence regarding the role of IL-5 in the development of AHR (15, 29), we show here that mL5.2 DNA vaccination can inhibit AHR in both OVA sensitization and T cell transfer models of experimental asthma.

### Production of Th2 cytokines is reduced after DNA vaccination with Th-modified IL-5

To further characterize the mechanism of the mIL-5.2 DNA vaccine-mediated inhibition of eosinophilia and AHR, we measured the production of various cytokines in the lungs and by OVA-stimulated peribronchial lymph node (PBLN) cells. No significant differences between naive nontreated controls and mIL-5.wt DNA-vaccinated mice were seen in the production of IL-4, IL-5, IL-10, or IL-13 at the level of protein or message production. By contrast, mIL-5.2 DNA-vaccinated mice had significant reductions in IL-5 protein (40–60%) and message levels (Tables II and III). Surprisingly, IL-4 and IL-10 production were also reduced at the level of protein and message after mIL-5.2 DNA vaccination. No significant reductions were seen in the production of OVA-specific IgE levels or IL-13 (Tables II and III and data not shown). These data suggest that active vaccination against murine IL-5 can reduce not only IL-5 levels, but also other key cytokines involved in the pathogenesis of asthma, presumably by reducing the numbers of inflammatory cells (lymphocytes and eosinophils; see Table I) recruited to the lung that are producing Th2 cytokines.

### Discussion

In this investigation, we show that active vaccination against IL-5 is a novel therapeutic approach for the treatment of asthma and potentially other eosinophilic disorders. Asthma as well as many other chronic diseases are associated with the aberrant expression of self-proteins. The expression of IL-5 in the lung is inversely correlated with pulmonary dysfunction in asthmatics, and the level of expression is directly correlated with the number of eosinophils detected in asthmatic airways (8, 10, 11). Animal studies have indicated that neutralizing IL-5 can profoundly attenuate eosinophilia and the subsequent damage caused by these leukocytes in the allergic lung (17). Reducing IL-5 levels can also reduce AHR independently of its role in eosinophilia (30), probably via the effects of IL-5 on airways smooth muscle (31, 32). The Th-modified IL-5 vaccine approach demonstrates the therapeutic potential for immunologically based vaccines directed against pathogenic self-proteins. By incorporating a promiscuous foreign Th epitope into self-proteins, thus providing sufficient T cell help, immunological tolerance to self-proteins can be bypassed (18–20). We show here that a murine Th-modified IL-5 DNA vaccine can induce an immune response that produces polyclonal Abs that are cross-reactive with native murine IL-5. Moreover, this immune

Table III. Reduced production of Th2 cytokines in PBLN cells of Th-modified IL-5 DNA-vaccinated OVA-sensitized mice<sup>a</sup>

	OVA <sup>b</sup>		
	IL-4 (ng/ml)	IL-5 (ng/ml)	IL-13 (ng/ml)
Saline	ND	ND	ND
OVA	0.77 ± 0.05 <sup>c</sup>	61 ± 3.3	15.5 ± 2.5
OVA/mIL5.wt	0.71 ± 0.06	54 ± 9.1	16 ± 3
OVA/mIL5.2	0.31 ± 0.03*	26 ± 4.3*	13 ± 0.1

<sup>a</sup> Cells from the PBLN were recovered from mice after exposure to an aerosol of OVA.

<sup>b</sup> Cells were stimulated with OVA in MLC medium for 72 h, and cytokine ELISAs were performed on supernatants.

<sup>c</sup> Mean cytokine production is shown (±SEM; *n* = 4 mice per group). Assays were repeated twice independently in duplicate.

\* IL-5 and IL-4, *p* < 0.05 for OVA-sensitized mIL-5.wt-vaccinated mice when compared with OVA-sensitized mIL-5.2-vaccinated mice.

response reduces airways inflammation, AHR and the production of key pathogenic Th2 cytokines in the pulmonary compartments of mice exposed to allergen-induced models of experimental asthma.

Immune responses against self-proteins such as IL-5 are usually not generated due to immunological tolerance. B cell Ag receptors to self-proteins are normally removed from the repertoire to induce tolerance and avoid autoimmunity (33, 34). However, immature B cells undergoing the induction of tolerance are exquisitely sensitive to T cell help, which, if provided, can rescue B cells from the induction of tolerance and promote B cell development (35). Importantly, we demonstrate that vaccination with Th-modified IL-5 DNA provides abundant T cell help that is capable of inducing a B cell response that elicits cross-reactive immunity with native IL-5. Our studies suggest that a portion of B cell tolerance is maintained by the careful regulation of T cell help. Indirectly, tolerance of the CD4 T cell compartment regulates B cell tolerance. Vaccination with DNA encoding Th epitope-modified self-proteins (but not DNA encoding unmodified self-proteins; see Fig. 2) that can provide T cell help in the appropriate context bypasses B cell tolerance.

Analysis of cytokine production in draining lymph nodes revealed a general reduction in Th2-type cytokines. Reduced levels of IL-5, IL-4, and IL-10 were measured in mIL-5.2-vaccinated

Table II. Reduced production and expression of Th2 cytokines in PBLN cells and lungs of Th-modified IL-5 DNA-vaccinated mice that received CD4<sup>+</sup> T cells<sup>a</sup>

	OVA <sup>b</sup>				
	IL-4 (ng/ml)	IL-5 (ng/ml)	IL-10 (ng/ml)	IL-13 (ng/ml)	IFN-γ (ng/ml)
OVA	0.57 ± 0.03 <sup>c</sup>	39 ± 3.0	27 ± 3.0	14 ± 1.7	ND
OVA/mIL5.wt	0.63 ± 0.04	41.2 ± 6.8	25 ± 0.2	13 ± 2.0	ND
OVA/mIL5.2	0.24 ± 0.03*	23.7 ± 2.0*	17 ± 2.0*	11 ± 0.3	ND
	(transcript U/HPRT)	(transcript U/HPRT)	(transcript U/HPRT)	(transcript U/HPRT)	(transcript U/HPRT)
OVA	0.72 ± 0.03 <sup>d</sup>	0.59 ± 0.03	0.56 ± 0.03	0.71 ± 0.03	0.15 ± 0.01
OVA/mIL5.wt	0.74 ± 0.02	0.65 ± 0.01	0.59 ± 0.03	0.78 ± 0.03	0.15 ± 0.01
OVA/mIL5.2	0.22 ± 0.19**	0.27 ± 0.02**	0.37 ± 0.02**	0.75 ± 0.04	0.13 ± 0.02

<sup>a</sup> Cells from the PBLN were recovered from mice after transfer of T cells and exposure to an aerosol of OVA.

<sup>b</sup> Cells were stimulated with OVA in MLC medium for 72 h, and cytokine ELISAs were performed on supernatants.

<sup>c</sup> Mean cytokine production is shown (±SEM; *n* = 4 mice per group). Assays were repeated twice independently in duplicate.

<sup>d</sup> Total RNA was isolated from lungs and RT-PCR was performed to determine relative quantities of mRNA for various cytokines. Values were derived by dividing the mean of the triplicate values measured for the transcript of interest by the mean of triplicate HPRT values for the sample (*n* = 4 mice per group).

\* IL-5, *p* < 0.05; IL-4, *p* < 0.005; IL-10, *p* < 0.05 for OVA-sensitized mIL-5.wt-vaccinated mice when compared with OVA-sensitized mIL-5.2-vaccinated mice.

\*\* IL-5 and IL-4, *p* < 0.005 for OVA-sensitized mIL-5.wt-vaccinated mice when compared with OVA-sensitized mIL-5.2-vaccinated mice.

mice. Previous studies have shown that administering short non-specific immunostimulatory DNA sequences could inhibit eosinophilia, IL-5, and AHR in similar models of allergen-induced lung disease (36, 37). The proposed mechanisms in those studies suggest that both an immediate production of IL-12 and IFN- $\gamma$  by the innate immune system and a general shift from a Th2 to a Th1 immune response by the adaptive immune system inhibit the activation of bone marrow-derived eosinophils and the subsequent generation of AHR. Although we cannot rule out a similar mechanism playing some role in the present studies, neither the mIL-5.wt nor the mIL-5.2 DNA vaccine induced a detectable Th1 immune response. IFN- $\gamma$  levels were consistently low in all study groups (Table II). Our data suggest that our vaccine approach is highly specific for targeting IL-5 and that the OVA-allergen induces a Th2 immune response as seen in the cytokine profile (see Tables II and III) and anti-OVA Ab isotypes (data not shown). DNA encoding wild-type murine IL-5 (or vector-only controls) showed no effect on the general Th2 environment and did induce Th1 cytokine production (Table II) or skew Ab isotypes. In addition, it is unlikely that a general Th1 environment could skew the cytokine production by the adoptively transferred OVA-specific Th2 CD4<sup>+</sup> T cells in such a short time frame. A more plausible explanation of the reduction in Th2 cytokine levels is that by neutralizing IL-5 and thus inhibiting T cell and eosinophil infiltrations into the lung, we have reduced the total number of cells capable of producing these Th2 cytokines (see Table I). The broad effects obtained by reducing IL-5 levels suggest that IL-5 production is central to a cascade of events that eventually results in the production of other Th2 cytokines and airways inflammation leading to AHR.

In summary, the Th-modified vaccine approach is capable of circumventing many of the problems associated with previous vaccine techniques, namely, bypassing immune tolerance and generating a polyclonal immune response. This approach also allows for longitudinal immunotherapy and potential resolution from chronic disease. Numerous studies have validated IL-5 as a therapeutic target for the treatment of asthma (8, 10, 11, 15, 38, 39), and promising results have been seen with anti-IL-5 mAb treatment in various animal models (16, 17, 40–46). Several clinical trials are currently evaluating the efficacy of anti-IL-5 mAb therapy in man, but have shown limited success to date in allergic asthmatics (47). Additional studies are required to determine the clinical efficacy of IL-5 neutralization in asthma as well as other eosinophilic disorders. By contrast to systemic humanized anti-IL-5 mAb administration, it is conceivable that in our vaccination approach, by using autologous B cells to deliver the therapeutic Abs at high concentration at the disease sites, we access microenvironments that are critical for disease expression and neutralize the pathogenic properties of IL-5. Although our DNA vaccine did not appear to drive a Th1 immune response, the ability of DNA vaccination to deviate immune responses toward a Th1 profile could be beneficial in treating allergy and could be exploited with our vaccine by adjusting the dose, route, or frequency of the DNA vaccine administration. Preliminary safety studies monitoring organ weights and general histology of selected tissues, including trachea and airways smooth muscle, show no differences between naive untreated and Th-modified IL-5 DNA-vaccinated mice (data not shown). Preliminary observations show that by 45–50 days after the last DNA vaccination Ab levels are declining (data not shown), as expected from previous vaccination studies with other Th-modified vaccines (A. Neisig, M. Hertz, and I. Dalum, unpublished observations). In conclusion, Th-modified IL-5 DNA provides a cost-effective therapeutic vaccine method for inhibiting pulmonary inflammation and AHR in response to allergen provocation of the airways. Active

vaccination against IL-5 represents a novel therapeutic approach for the treatment of asthma and other allergic conditions.

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## References

- De Monchy, J. G., H. F. Kauffman, P. Venge, G. H. Koeter, H. M. Jansen, H. J. Sluiter, and K. De Vries. 1985. Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. *Am. Rev. Respir. Dis.* 131:373.
- Beasley, R., W. R. Roche, J. A. Roberts, and S. T. Holgate. 1989. Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am. Rev. Respir. Dis.* 139:806.
- Azzawi, M., B. Bradley, P. K. Jeffery, A. J. Frew, A. J. Wardlaw, G. Knowles, B. Assoufi, J. V. Collins, S. Durham, and A. B. Kay. 1990. Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. *Am. Rev. Respir. Dis.* 142:1407.
- Ohashi, Y., S. Motojima, T. Fukuda, and S. Makino. 1992. Airway hyperresponsiveness, increased intracellular spaces of bronchial epithelium, and increased infiltration of eosinophils and lymphocytes in bronchial mucosa in asthma. *Am. Rev. Respir. Dis.* 145:1469.
- Nakajima, H., I. Iwamoto, S. Tomoe, R. Matsumura, H. Tomioka, K. Takatsu, and S. Yoshida. 1992. CD4<sup>+</sup> T-lymphocytes and interleukin-5 mediate antigen-induced eosinophil infiltration into the mouse trachea. *Am. Rev. Respir. Dis.* 146:374.
- Broide, D. H., G. J. Gleich, A. J. Cuomo, D. A. Coburn, E. C. Federman, L. B. Schwartz, and S. I. Wasserman. 1991. Evidence of ongoing mast cell and eosinophil degranulation in symptomatic asthma airway. *J. Allergy Clin. Immunol.* 88:637.
- Wardlaw, A. J., S. Dunnette, G. J. Gleich, J. V. Collins, and A. B. Kay. 1988. Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma: relationship to bronchial hyperactivity. *Am. Rev. Respir. Dis.* 137:62.
- Robinson, D. S., S. Ying, A. M. Bentley, Q. Meng, J. North, S. R. Durham, A. B. Kay, and Q. Hamid. 1993. Relationships among numbers of bronchoalveolar lavage cells expressing messenger ribonucleic acid for cytokines, asthma symptoms, and airway methacholine responsiveness in atopic asthma. *J. Allergy Clin. Immunol.* 92:397.
- Walker, C., M. K. Kacgi, P. Braun, and K. Blaser. 1991. Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. *J. Allergy Clin. Immunol.* 88:935.
- Robinson, D. S., Q. Hamid, S. Ying, A. Tsicopoulos, J. Barkans, A. M. Bentley, C. Corrigan, S. R. Durham, and A. B. Kay. 1992. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N. Engl. J. Med.* 326:298.
- Robinson, D., Q. Hamid, A. Bentley, S. Ying, A. B. Kay, and S. R. Durham. 1993. Activation of CD4<sup>+</sup> T cells, increased TH2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. *J. Allergy Clin. Immunol.* 92:313.
- Walker, C., E. Bode, L. Boer, T. T. Hansel, K. Blaser, and J. C. Virchow, Jr. 1992. Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am. Rev. Respir. Dis.* 146:109.
- Drazen, J. M., J. P. Arm, and K. F. Austen. 1996. Sorting out the cytokines of asthma. *J. Exp. Med.* 183:1.
- Bousquet, J., P. Chanez, J. Y. Lacoste, G. Barneon, N. Ghavanian, I. Enander, P. Venge, S. Ahlstedt, J. Simony-Lafontaine, P. Godard, et al. 1990. Eosinophilic inflammation in asthma. *N. Engl. J. Med.* 323:1033.
- Foster, P. S., S. P. Hogan, A. J. Ramsay, K. I. Matthaci, and I. G. Young. 1996. Interleukin 5 deficiency abolishes eosinophilia, airways hyperactivity, and lung damage in a mouse asthma model. *J. Exp. Med.* 183:195.
- Mausser, P. J., A. M. Pitman, X. Fernandez, S. K. Foran, G. K. Adams III, W. Kreutner, R. W. Egan, and R. W. Chapman. 1995. Effects of an antibody to interleukin-5 in a monkey model of asthma. *Am. J. Respir. Crit. Care Med.* 152:467.
- Mausser, P. J., A. Pitman, A. Witt, X. Fernandez, J. Zurcher, T. Kung, H. Jones, A. S. Watnick, R. W. Egan, W. Kreutner, et al. 1993. Inhibitory effect of the TRFK-5 anti-IL-5 antibody in a guinea pig model of asthma. *Am. Rev. Respir. Dis.* 148:1623.
- Dalum, I., D. M. Butler, M. R. Jensen, P. Hindersson, L. Steinnaa, A. M. Waterston, S. N. Grell, M. Feldmann, H. I. Elsner, and S. Mouritsen. 1999. Therapeutic antibodies elicited by immunization against TNF- $\alpha$ . *Nat. Biotechnol.* 17:666.
- Dalum, I., M. R. Jensen, P. Hindersson, H. I. Elsner, and S. Mouritsen. 1996. Breaking of B cell tolerance toward a highly conserved self protein. *J. Immunol.* 157:4796.
- Dalum, I., M. R. Jensen, K. Gregorius, C. M. Thomasen, H. I. Elsner, and S. Mouritsen. 1997. Induction of cross-reactive antibodies against a self protein by immunization with a modified self protein containing a foreign T helper epitope. *Mol. Immunol.* 34:1113.
- Kozak, M. 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* 15:8125.

22. Panina-Bordignon, P., A. Tan, A. Termijtelen, S. Demotz, G. Corradin, and A. Lanzavecchia. 1989. Universally immunogenic T cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T cells. *Eur. J. Immunol.* 19:2237.
23. Hogan, S. P., K. I. Matthaei, J. M. Young, A. Koskinen, I. G. Young, and P. S. Foster. 1998. A novel T cell-regulated mechanism modulating allergen-induced airways hyperactivity in BALB/c mice independently of IL-4 and IL-5. *J. Immunol.* 161:1501.
24. Hamelmann, E., J. Schwarze, K. Takeda, A. Oshiba, G. L. Larsen, C. G. Irvin, and E. W. Gelfand. 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* 156:766.
25. Webb, D. C., A. N. McKenzie, A. M. Koskinen, M. Yang, J. Mattes, and P. S. Foster. 2000. Integrated signals between IL-13, IL-4, and IL-5 regulate airways hyperactivity. *J. Immunol.* 165:108.
26. Wynn, T. A., I. Eltoum, A. W. Cheever, F. A. Lewis, W. C. Gause, and A. Sher. 1993. Analysis of cytokine mRNA expression during primary granuloma formation induced by eggs of *Schistosoma mansoni*. *J. Immunol.* 151:1430.
27. Stamm, L. M., A. Raisanen-Sokolowski, M. Okano, M. E. Russell, J. R. David, and A. R. Satoskar. 1998. Mice with STAT6-targeted gene disruption develop a Th1 response and control cutaneous leishmaniasis. *J. Immunol.* 161:6180.
28. Hogan, S. P., A. Koskinen, K. I. Matthaei, I. G. Young, and P. S. Foster. 1998. Interleukin-5-producing CD4<sup>+</sup> T cells play a pivotal role in aeroallergen-induced eosinophilia, bronchial hyperactivity, and lung damage in mice. *Am. J. Respir. Crit. Care Med.* 157:210.
29. Corry, D. B., H. G. Folkesson, M. L. Warnock, D. J. Erle, M. A. Matthay, J. P. Wiener-Kronish, and R. M. Locksley. 1996. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperactivity. *J. Exp. Med.* 183:109.
30. Toumoy, K. G., J. C. Kips, and R. A. Pauwels. 2001. The allergen-induced airway hyperresponsiveness in a human-mouse chimera model of asthma is T cell and IL-4 and IL-5 dependent. *J. Immunol.* 166:6982.
31. Hakonarson, H., N. Maskeri, C. Carter, and M. M. Grunstein. 1999. Regulation of TH1- and TH2-type cytokine expression and action in atopic asthmatic sensitized airway smooth muscle. *J. Clin. Invest.* 103:1077.
32. Hakonarson, H., N. Maskeri, C. Carter, S. Chuang, and M. M. Grunstein. 1999. Autocrine interaction between IL-5 and IL-1 $\beta$  mediates altered responsiveness of atopic asthmatic sensitized airway smooth muscle. *J. Clin. Invest.* 104:657.
33. Hertz, M., and D. Nemazee. 1998. Receptor editing and commitment in B lymphocytes. *Curr. Opin. Immunol.* 10:208.
34. Goodnow, C. C. 1996. Balancing immunity and tolerance: deleting and tuning lymphocyte repertoires. *Proc. Natl. Acad. Sci. USA* 93:2264.
35. Buhl, A. M., D. Nemazee, J. C. Cambier, R. Rickert, and M. Hertz. 2000. B-cell antigen receptor competence regulates B-lymphocyte selection and survival. *Immunol. Rev.* 176:141.
36. Broide, D., J. Schwarze, H. Tighe, T. Gifford, M. D. Nguyen, S. Malek, J. Van Uden, E. Martin-Orozco, E. W. Gelfand, and E. Raz. 1998. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J. Immunol.* 161:7054.
37. Kline, J. N., T. J. Waldschmidt, T. R. Businga, J. E. Lemish, J. V. Weinstock, P. S. Thorne, and A. M. Krieg. 1998. Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J. Immunol.* 160:2555.
38. Sur, S., G. J. Gleich, K. P. Offord, M. C. Swanson, T. Ohnishi, L. B. Martin, J. M. Wagner, D. A. Weiler, and L. W. Hunt. 1995. Allergen challenge in asthma: association of eosinophils and lymphocytes with interleukin-5. *Allergy* 50:891.
39. Ohnishi, T., H. Kita, D. Weiler, S. Sur, J. B. Sedgwick, W. J. Calhoun, W. W. Busse, J. S. Abrams, and G. J. Gleich. 1993. IL-5 is the predominant eosinophil-active cytokine in the antigen-induced pulmonary late-phase reaction. *Am. Rev. Respir. Dis.* 147:901.
40. Egan, R. W., D. Athwahl, C. C. Chou, S. Emtage, C. H. Jehn, T. T. Kung, P. J. Mauser, N. J. Murgolo, and M. W. Bodmer. 1995. Inhibition of pulmonary eosinophilia and hyperactivity by antibodies to interleukin-5. *Int. Arch. Allergy Immunol.* 107:321.
41. Van Oosterhout, A. J., A. R. Ladenius, H. F. Savelkoul, I. Van Ark, K. C. Delsman, and F. P. Nijkamp. 1993. Effect of anti-IL-5 and IL-5 on airway hyperactivity and eosinophils in guinea pigs. *Am. Rev. Respir. Dis.* 147:548.
42. Akutsu, I., T. Kojima, A. Kariyone, T. Fukuda, S. Makino, and K. Takatsu. 1995. Antibody against interleukin-5 prevents antigen-induced eosinophil infiltration and bronchial hyperactivity in the guinea pig airways. *Immunol. Lett.* 45:109.
43. Gulbenkian, A. R., R. W. Egan, X. Fernandez, H. Jones, W. Kreutner, T. Kung, F. Payvand, L. Sullivan, J. A. Zurcher, and A. S. Watnick. 1992. Interleukin-5 modulates eosinophil accumulation in allergic guinea pig lung. *Am. Rev. Respir. Dis.* 146:263.
44. Kung, T. T., D. M. Stelts, J. A. Zurcher, G. K. Adams III, R. W. Egan, W. Kreutner, A. S. Watnick, H. Jones, and R. W. Chapman. 1995. Involvement of IL-5 in a murine model of allergic pulmonary inflammation: prophylactic and therapeutic effect of an anti-IL-5 antibody. *Am. J. Respir. Cell Mol. Biol.* 13:360.
45. Shardonofsky, F. R., J. Venzor III, R. Barrios, K. P. Leong, and D. P. Huston. 1999. Therapeutic efficacy of an anti-IL-5 monoclonal antibody delivered into the respiratory tract in a murine model of asthma. *J. Allergy Clin. Immunol.* 104:215.
46. Nagai, H., S. Yamaguchi, N. Inagaki, N. Tsuruoka, Y. Hitoshi, and K. Takatsu. 1993. Effect of anti-IL-5 monoclonal antibody on allergic bronchial eosinophilia and airway hyperresponsiveness in mice. *Life Sci.* 53:L243.
47. Leckie, M. J., A. ten Brinke, J. Khan, Z. Diamant, B. J. O'Connor, C. M. Walls, A. K. Mathur, H. C. Cowley, K. F. Chung, R. Djukanovic, et al. 2000. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyperresponsiveness, and the late asthmatic response. *Lancet* 356:2144.

# emerging therapeutic vaccines

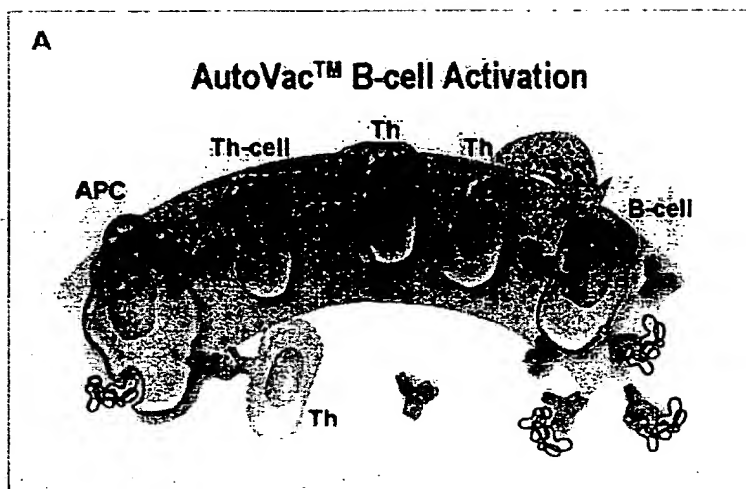
The excitement over the past 30 years for immunotherapy of cancer and other diseases has not led to the expected clinical successes. Over-enthusiasts predicted a cure for cancer with the initial development of monoclonal antibody technology, and later the 'magic bullets' or toxin-labelled antibodies. Identification of proteins restricted to, or at least overexpressed in tumours has also led to disappointing clinical results. The main barriers have been a lack of immunological understanding of the processes at work, eg immune tolerance. Advances in our understanding of how to induce strong immune responses and how to manipulate the immune system to avoid immunological tolerance have opened the way for emerging therapeutic vaccinations in the treatment of not only cancer, but other diseases as well. This review will focus on immunotherapy for cancer and chronic human diseases characterised by the altered expression of self-proteins.

**I**mmunological tolerance regulates the immune system such that foreign pathogens are quickly attacked, while immune responses directed against the body are rare. This is achieved by functionally removing cells that recognise self-antigens from the immune system. In most cases, a breakdown of immune tolerance is undesirable, and leads to autoimmunity. However, in some situations it is beneficial to elicit an immune response against self-proteins aberrantly expressed in chronic human diseases and in cancer (eg the overexpression of Her-2 in breast cancer). The immune system is divided into two main responses, the innate immune response and the adaptive immune response. The innate immune response is the body's first line of defence and is mostly nonspecific; while the adaptive immune response is a specific response to the invading pathogens, and is the response targeted by vaccination. The adaptive response is composed of B and T lymphocytes that recognise specific 'epitopes' or structures of the pathogen. B lymphocytes recognise three-dimensional structures on proteins and produce antibodies that bind these structures. T lymphocytes

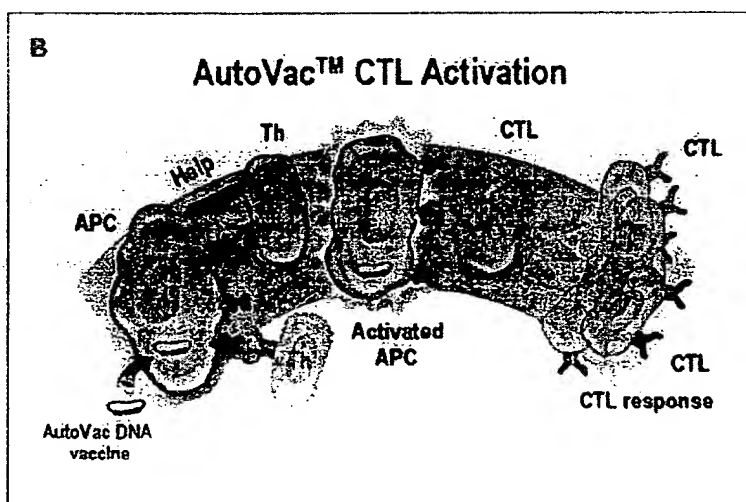
recognise short peptides (8-15 mers), presented in the context of the major histocompatibility complex (MHC) on antigen presenting cells, and become activated. T helper (Th) cells recognise peptides presented in MHC class II complexes and provide signals that help to activate other cells. Cytotoxic T lymphocytes (CTLs) recognise peptides presented in MHC class I complexes and kill target cells that present the same peptide/MHC class I complexes.

Considerable excitement concerning the treatment and diagnosis of disease, mainly cancer, arose with the development of monoclonal antibody technology in 1975 by Kohler and Milstein<sup>1</sup>. Unfortunately, very little clinical progress has been made utilising monoclonal antibodies to treat disease, with several noted exceptions (Rituximab ( $\alpha$ -CD20), Remicade ( $\alpha$ -TNF) and Herceptin ( $\alpha$ -Her-2)). Part of the failure of monoclonal antibodies in treating disease may partly be due to the lack of support from the pharmaceutical industry, which pulled its R&D funding after initial endeavours did not live up to expectations. However, there has been a resurgence of interest and success with monoclonal

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**Figure 1**  
AutoVac™ can bypass immunological tolerance by providing help to B and T lymphocytes. The AutoVac™ recombinant protein, or DNA, is taken up, processed and peptides are presented by MHC class II on antigen presenting cells (APC). Simultaneously, the T helper (Th) cells that recognise the promiscuous Th epitopes presented by MHC class II molecules, and the APC become activated, upregulate costimulatory molecules and produce cytokines. **A** B-cells recognising the self-antigen also internalise the AutoVac™ protein via the B cell antigen receptor, process and present the peptides to Th cells. Activated Th cells that recognise the peptides presented by B-cells activate the B-cells to differentiate into antibody secreting plasma cells that produce antibody capable of crossreacting with the non-modified self-protein. **B** The activation of APCs by Th cells is required to stimulate CTLs, recognising peptides bound to MHC class I molecules, capable of killing target cells expressing the self-protein.



antibodies, which is mirrored in the number of monoclonal antibodies marketed or in late stage clinical trials for both cancer and immune system disorders such as asthma, allergy and autoimmunity (see Table 1).

The treatment efficacy of a monoclonal antibody is usually lower than that of a strong polyclonal response, and the high doses and frequent patient administrations can cause problems. Most notably, endogenous immune responses to the 'foreignness' of the monoclonal antibody in the form of anti-idiotypic antibodies and immune reactions to xenogenic and isotypic aspects of the monoclonal antibody if it is not fully humanised have caused significant problems. The recent generation of mice expressing human immunoglobulin gene segments should facilitate the production of fully humanised monoclonal antibodies<sup>2</sup>. In addition to producing humanised monoclonal antibodies, much improved preclinical data can be obtained using these mice in combination with mice expressing human major histocompatibility complex (MHC) molecules<sup>3-5</sup>. Better immunological understanding of how monoclonal antibodies function *in vivo* has greatly increased their success in therapy; however, therapeutic vaccinations may have overwhelming advantages to monoclonal antibody therapy (see Table 2).

Active vaccination has the advantage of recruiting the entire immune response to subdue the targeted disease. Vaccination can induce a polyclonal antibody response that generates antibodies to several epitopes and of different isotypes<sup>6</sup>. The efficacy of clearing a soluble antigen is arguably increased by a polyclonal antibody response compared to the ability of a monoclonal antibody. In addition, the activation of antibody-dependent effector functions, the key to the success of antibodies in cancer immunotherapy, is orders of magnitude better with a polyclonal antibody response than with a monoclonal antibody. In fact, the inability of monoclonal antibodies to activate effector functions can explain some of the poor results obtained in previous cancer trials. In addition to the humoral response, vaccination has the advantage of activating cell-mediated immunity such as CTLs. Activated CTLs can directly kill other cells, such as a tumour, expressing the target antigen and MHC class I. The advantages of inducing both a polyclonal humoral response and a strong cell-mediated response would provide a new generation of therapeutic vaccinations with a greater chance of clinical success.

The first therapeutic vaccines utilised donor tumour cells as immunogens. Irradiated devitalised tumour cells are injected back into patients with the aim of raising an immune response to the tumour. Tolerance may prohibit immune responses to the dominant epitopes, but by providing some T cell help



**Table 1**  
**Monoclonal antibodies and therapeutic vaccines in clinical trials<sup>a</sup>**

<b>CANCER</b>			
<b>Monoclonal antibodies</b>			
Anti-EGFR (C225)	ImClone Systems	Phase III	head & neck
Anti-EGFR + cisplatin (C225)	ImClone Systems	Phase I/II	non-small cell lung
Lym-15131 (Oncozym)	Techniclone International	Phase III	non-Hodgkin's lymphoma
Anti-CD20-131 (Bexcar)	Coulter Pharmaceuticals	FDA action	non-Hodgkin's lymphoma
Anti-CD33 (SMART-M195)	Protein Design Labs	Phase III <sup>b</sup>	acute myeloid leukemia
Anti-VEGF	Genentech	Phase III <sup>b</sup>	colon & non-small cell lung
Anti-CD52 (Campath)	Ilex Oncology	PLA/ND <sup>b</sup>	chronic lymphocytic leukemia
Anti-CD52 (Campath)	LeukoSite	PLA/ND <sup>b</sup>	chronic lymphocytic leukemia
Anti-CD33 + cytotoxin (CMA676)	Celltech Group	Phase II/III	acute myeloid leukemia
Anti-CD33 (AHP)	Celltech Group	PLA/ND <sup>b</sup>	acute myeloid leukemia
Anti-CD20 (Mabthera/Rituxan)	IDEC Pharmaceuticals	Marketed	non-Hodgkin's lymphoma
Anti-Her-2 (Herceptin)	Genentech	Marketed	breast & ovarian
Anti-CD20 + yttrium <sup>90</sup> (Zevalin)	IDEC Pharmaceuticals	Phase III <sup>b</sup>	non-Hodgkin's lymphoma
<b>Vaccines</b>			
BCG vaccine (Avivert)	AVI Biopharma	Phase III <sup>b</sup>	bladder cancer
ICG vaccine (Avivert)	AVI Biopharma	Phase III <sup>b</sup>	colon
CEA vaccine (CesVax)	Intan	Phase III <sup>b</sup>	colorectal
GM2 conjugate vaccine (GMK)	Progenics	Phase III <sup>b</sup>	melanoma
T-cell vaccine + GM-CSF gene (GVAX)	Cell Genesys	Phase III <sup>b</sup>	melanoma
Melanoma vaccine (Melacine)	RIBI Immunochem	PLA/ND <sup>b</sup>	melanoma
Melanoma vaccine (Melacine)	RIBI Immunochem	Phase III <sup>b</sup>	breast cancer
OncoVax <sup>®</sup>	Intracell	PLA/ND <sup>b</sup>	colon
Gastrin 17 peptides (Gastrimmune)	Aphtron	Phase III <sup>b</sup>	pancreatic
Gastrin 17 peptides (Gastrimmune)	Aphtron	PLA/ND <sup>b</sup>	gastrointestinal
Hapten-modified tumour cell (M-vax)	Avax Technologies	Phase III <sup>b</sup>	melanoma
<b>ASTHMA, ALLERGY, AUTOIMMUNITY, INFLAMMATION &amp; TRANSPLANTATION</b>			
<b>Monoclonal antibodies</b>			
Anti-TNF $\alpha$ (D2E7)	Cambridge Antibody	Phase III	rheumatoid arthritis
Anti-TNF $\alpha$ (Remicade)	Centocor	Marketed	rheumatoid arthritis & Crohn's disease
Anti-TNF $\alpha$ (Norasept)	CellTech Group	Phase III <sup>b</sup>	Crohn's disease
Anti-CD20 (Zenapax)	Protein Design Labs	Marketed	kidney transplantation
Anti-IgE (E25)	Tanox	Phase III <sup>b</sup>	asthma & allergic rhinitis
Anti-IgE (E25)	Tanox	Phase III	allergy
Anti-IgE	Genentech	Phase III <sup>b</sup>	asthma & allergic rhinitis
Anti-leukointegrin (LeukoArrest)	ICOS	Phase III	ischemic stroke
Anti-T cell (MEDI-500)	Medimmune	Phase III	graft versus host disease
<b>Vaccines</b>			
TCR-peptide vaccine (I)	Immune Response	Phase III <sup>b</sup>	rheumatoid arthritis
MHC peptide (Anervax)	Anergen (Corixa)	Phase II	rheumatoid arthritis
Heat-killed M. vaccae extract (PVAX)	Corixa	Phase II	psoriasis

<sup>a</sup> Clinical trial data taken from [www.recap.com](http://www.recap.com), and is not presented as a complete listing

<sup>b</sup> Product licence application/new drug application

<sup>c</sup> Approved for sale in Canada

## References

- Kohler, G and Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256 (5517):495-497, 1975.
- Bruggemann, M and Taussig, MJ. Production of human antibody repertoires in transgenic mice. *Curr Opin Biotechnol* 8 (4):455-458, 1997.
- Abraham, RS and David, CS. Identification of HLA-class-II-restricted epitopes of autoantigens in transgenic mice. *Curr Opin Immunol* 12 (1):122-129, 2000.
- Arnold, B and Hammerling, GJ. MHC class-I transgenic mice. *Annu Rev Immunol* 9:297-322, 1991.
- Taneja, V and David, CS. HLA class II transgenic mice as models of human diseases. *Immunol Rev* 169:67-79, 1999.
- Dalrymple, B, Butler, DM, Jensen, MR, Hindersson, P, Steinha, L, Waterston, AM, Grell, SN, Feldmann, M, Elsnher, H, and Mouritsen, S. Therapeutic antibodies elicited by immunisation against TNF- $\alpha$ . *Nat Biotechnol* 17 (7):666-669, 1999.
- Rosenberg, SA. *Cell Transfer Therapy*. Philadelphia: Lipincott, 1995.
- Blachere, NE, Udono, H, Janetzki, S, Li, Z, Heike, M, and Srivastava, PK. Heat shock protein vaccines against cancer. *J Immunother* 14 (4):352-356, 1993.
- Ludewig, B, Ochsenbein, AF, Odermatt, B, Paulin, D, Hengartner, H, and Zinkernagel, RM. Immunotherapy with dendritic cells directed against tumour antigens shared with normal host cells results in severe autoimmune disease. *J Exp Med* 191 (5):795-804, 2000.
- Herzenberg, LA and Tokuhisa, T. Carrier-priming leads to hapten-specific suppression. *Nature* 285 (5767):664-667, 1980.

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**Table 2**  
**Comparison of monoclonal antibody therapy to other vaccination methods**

	MONOCLONAL ANTIBODY	CONJUGATE VACCINE	CELLULAR VACCINES	PEPTIDE VACCINES	VIRAL VECTOR	AUTOVAC
Dose Required	High	Low to moderate	High	High	Low	Low
Duration/Title	Weeks	Months	Months	Months	Months	Months
Efficacy	Moderate to High	Low-Moderate	Low	Moderate	High	High
Antibody Secondary Effector Functions	Low	High	High	Low	High	High
Immune Response to Dominant vs Subdominant Epitopes	Single Epitope	Subdominant	Subdominant	Subdominant	Both	Both
Antibody/Fine Specificity	Monospecific	Variable	Variable	Limited	Variable	Polyspecific
Carrier/Viral/Auto-Idiotype Suppression	Yes	Yes	No	Yes	Yes	No
Cell-mediated Immunity	Not Applicable	Low	Low	High	High	High
Hospitalisation	Required	None	Required	None	None	None
Production Costs	High	Low	High	Low	Low	Low
Safety Characterisation	Easily Characterised	Difficult to Characterise	Difficult to Characterise	Easily Characterised	Difficult to Characterise	Easily Characterised

in the form of recombinant cytokines, viral oncolysates or genetically modified tumour cells (eg to express higher levels of MHC and co-stimulatory molecules) limited success can be achieved<sup>7</sup>.

With the identification of tumour-associated antigens, peptide-based vaccinations can be developed to target specific antigens aberrantly expressed in cancer. Peptides are either obtained directly by acid-elution from MHC molecules on the cell surface of tumours (that can then be sequenced), or indirectly acquired from tumour-specific heat shock proteins (HSPs) that chaperone a wide variety of peptides<sup>8</sup>. Unfortunately, these approaches have the disadvantage of being expensive and labour intensive. In addition, because a complete set of peptides derived from both normal and tumour-associated proteins are included, development of autoimmunity to normal tissue may occur<sup>9</sup>. Determining the peptide specificity of tumour-specific CTLs *ex vivo* can be used to generate tumour-specific peptide vaccines for the generation of CTL responses. Peptides themselves are poorly immunogenic, and need to be conjugated

to a carrier protein. Conjugate vaccines are able to elicit immune responses to poorly immunogenic peptides by providing T-cell help in the form of a highly immunogenic carrier protein such as keyhole limpet haemocyanin (KLH). Unfortunately, the dominating immune response to the carrier protein results in a poor immune response to the target peptide<sup>10</sup>. Additionally, conjugated peptides may have problems entering the MHC class I presentation pathway for CTL activation. The other disadvantage of peptide-based therapies is that the immune response elicited is to that single peptide epitope, whereas a given protein probably contains several MHC class I and II binding peptides.

Other methods of employing peptide-based therapies include pulsing peptides onto dendritic cells that specialise in presenting antigen to B and T lymphocytes. Bone marrow derived dendritic cells are harvested from the patient, peptide is loaded on to the MHC molecules on the surface of the dendritic cell and the cells are reintroduced into the patient. The advantage of this approach is that the tumour-specific

Continued from page 51

ic peptides are efficiently 'presented' to the immune system in a way that optimally activates T cells. Multiple target epitopes can be presented to the dendritic cells with a cocktail containing several disease-associated peptides. This approach is limited by cost and efficacy: having to develop specific vaccines for each patient and bypassing immune tolerance to the peptide epitopes.

Therapeutic vaccines have been hindered by their inability to break immunological tolerance and to generate a robust response composed of both a humoral and a cellular immune component. Several recent advances have addressed these problems, including recombinant viral vaccinations, polynucleotide (RNA and DNA) vaccinations, and autovaccinations (AutoVac<sup>TM</sup>). All of these techniques can induce immune responses to conserved self-proteins by providing exogenous T cell help to lymphocytes, albeit at varying efficiencies.

Recombinant vaccinia virus can induce strong immune responses, both humoral and cell-mediated, to weakly immunogenic self-proteins. Recombinant viruses can be engineered to co-express multiple peptide epitopes<sup>11</sup>, immunostimulatory cytokines (such as IL-12) or costimulatory molecules (such as B7 family members). The extreme immunogenicity of vaccinia virus limits its use to a single vaccination that cannot be boosted<sup>12</sup>. The inability to boost or vaccinate with subsequent vaccinia-based therapies, and other safety concerns, limit the use of recombinant viral-based vaccinations.

Polynucleotide vaccinations are able to generate both a humoral and a cell-mediated immune response, but appear to favour cell-mediated immunity. Recombinant DNA or RNA is introduced into the patient, where it is expressed in either antigen presenting cells that directly activate lymphocytes; or it is expressed and released by some other cell type and the protein is sequestered by the antigen presenting cell. Preclinical data are promising, but more time is needed to generate clinical data in humans and to resolve safety concerns. RNA vaccinations have safety advantages over DNA vaccinations, in that RNA cannot incorporate into the host genome and has a shorter half-life than DNA.

Autovaccination is another approach that can bypass immunological tolerance. Rapid and robust humoral and cellular immune responses can be induced to conserved self-proteins by providing exogenous T cell help to self-reactive lymphocytes. By constructing proteins containing promiscuous foreign T cell epitopes inserted into flexible regions of self-proteins, therapeutic autovaccination can trick the immune system into responding to otherwise immunologically tolerated proteins. The mech-

anism is shown in Figure 1. This therapeutic vaccination approach utilises the basic concepts of B-T lymphocyte collaboration to induce self-reactive antibodies and Th cell activation of antigen presenting cells to stimulate CTLs<sup>13-17</sup>. Self-proteins carrying promiscuous foreign Th epitopes are endocytosed by antigen presenting cells and the epitopes are presented to Th cells by MHC class II molecules expressed on the antigen presenting cell. Activated Th cells then provide help (eg cytokines) to those B cells that have also internalised the modified self-proteins via the B cell antigen receptor; and antigen-specific B cells then secrete antibodies capable of cross-reacting with non-modified self-protein (Figure 1A). Using the appropriate delivery system, or DNA vaccination (Figure 1B), AutoVac<sup>TM</sup> molecules can also induce CTLs. As in Figure 1A, internalised peptide epitopes induce Th cells that then activate antigen presenting cells via CD40-CD40L interactions, for example. Activated antigen presenting cells can stimulate naive CTLs capable of killing target cells displaying tumour-specific peptides presented by MHC class I molecules. This approach has the advantages of bypassing immunological tolerance and activating both arms of the adaptive immune system to target self-antigens aberrantly expressed in disease, while avoiding several of the disadvantages of other therapeutic vaccine technologies (eg carrier suppression and peptide therapies, see Table 2). DDW

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11 Mateo, L, Gardner, J, Chen, Q, Schmidt, C, Down, M, Elliott, SL, Pye, SJ, Firat, H, Lemonnier, FA, Cebon, J, and Suhrbier, A. An HLA-A2 polypeptide vaccine for melanoma immunotherapy. *J Immunol* 163 (7): 4058-4063, 1999.

12 Kundig, TM, Kalberer, CP, Hengartner, H, and Zinkernagel, RM. Vaccination with two different vaccinia recombinant viruses: long-term inhibition of secondary vaccination. *Vaccine* 11 (11):1154-1158, 1993.

13 Dalum, I, Jensen, MR, Hindersson, P, Elner, H, and Mouritsen, S. Breaking of B cell tolerance toward a highly conserved self protein. *J Immunol* 157 (11):4796-4804, 1996.

14 Lanzavecchia, A. Immunology: Licence to kill [news comment]. *Nature* 393 (6684):413-414, 1998.

15 Ridge, JP, Di Rosa, F, and Matzinger, P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell [see comments]. *Nature* 393 (6684):474-478, 1998.

16 Schoenberger, SP, Toes, RE, der van, Offringa, VR, and Melief, CJ. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions [see comments]. *Nature* 393 (6684):480-483, 1998.

17 Bennett, SR, Carbone, FR, Karamalis, F, Flavell, RA, Miller, JF, and Heath, WR. Help for cytotoxic T-cell responses is mediated by CD40 signalling [see comments]. *Nature* 393 (6684):478-480, 1998.



## F400

**Prevention of Early Postmenopausal Bone Loss by Strontium Ranelate: A Randomised, Two-Year, Double-Blind, Placebo-Controlled Trial.** J. L. Reginster,<sup>1</sup> R. Deroisy,<sup>1</sup> Y. Toudouros,<sup>2</sup> L. Jupsin,<sup>1</sup> C. Roux.<sup>3</sup> <sup>1</sup>Bone and Cartilage Metabolism Unit, University of Liège, Liège, Belgium, <sup>2</sup>Institut de Recherches Internationales Servier, Courbevoie, France, <sup>3</sup>Rheumatology, Hôpital Cochin, Paris, France.

Postmenopausal bone loss remains a major public health problem because two-thirds of women do not want to receive, or cannot be treated with, hormone replacement therapy. Strontium ranelate is a novel agent that increases bone formation and appears to uncouple the processes of bone formation and resorption. One hundred and sixty healthy, early postmenopausal women were randomised to receive placebo or strontium ranelate (PROTOS®) 125 mg/day, 500 mg/day or 1 g/day for 2 years (40 participants per group). All participants received a calcium supplement of 500 mg daily. The primary efficacy parameter was annual increase in lumbar bone mineral density (BMD), measured using dual-energy X-ray absorptiometry. Secondary efficacy parameters included biochemical markers of bone turnover. At month 24, only strontium ranelate 1 g/day demonstrated a significantly greater increase in adjusted lumbar BMD than placebo ( $p < 0.05$ ). The annual increase was 0.7%, compared with -0.5% with placebo, with an overall beneficial effect after 2 years of about 2.4% in the strontium ranelate 1 g/day group compared with the placebo group. There were no other significant between-group differences in adjusted lumbar BMD. During treatment with strontium ranelate 1 g/day, a trend toward increased levels of bone formation markers was observed, with no effect on markers of bone resorption. Strontium ranelate displayed a tolerability profile similar to that of placebo. The minimum dose at which strontium ranelate is effective in preventing bone loss in early postmenopausal women is 1 g/day. This dose produced significantly greater increases in BMD than placebo, and changes in bone turnover markers were consistent with the exertion of an uncoupling effect on bone turnover. Further studies are now required to assess the efficacy of strontium ranelate in patients with established osteoporosis.

## F404

**Growth Hormone Increases Bone Mineral Content in Postmenopausal Osteoporosis.** K. L. Landin-Wilhelmsen, A. Nilsson. Endocrine Division, Dept. of Medicine, Göteborg, Sweden.

Growth hormone (GH) is an important regulator of bone. The aim was to study the effect of GH in osteoporotic, postmenopausal women. Eighty women, 50-70 years,  $\geq 5$  years after the menopause and ongoing estrogen therapy since  $\geq 9$  months were randomized to GH (Genotropin®), 1.0 U or 2.5 U/day vs similar amounts of placebo, subcutaneously, double-blind during 18 months. The placebo group then stopped the injections, but both GH groups continued another 18 months, in total 3 years with GH. Calcium 750 mg/day and vitamin D 400 U/day were given to all. Both the placebo group and the 2 GH groups are now followed-up until 4 years, i.e. one year after the GH treatment was terminated, but with continued estrogen and calcium/vitamin D supplementation. The 5-year follow-up will be completed in June 2001. Insulin-like growth factor-1 (IGF-1) and lean body mass increased dose dependently in both GH groups at 3 years ( $p < 0.001$ ) and decreased to pre-treatment levels at 4 years. Lumbar, femoral and areal bone mineral density and bone mineral content (BMC) increased 1-8% in all women, but no differences between the groups were seen after 3 years. Lumbar spine BMC increased 14% on 2.5 U GH and was higher than placebo ( $p = 0.0006$ ) at 4 years. Femur neck BMC increased 13% on 2.5 U GH and was higher than on 1.0 U GH ( $p = 0.01$ ). Total body BMC was higher on 2.5 U GH than on 1.0 U GH ( $p = 0.05$ ) and placebo ( $p = 0.01$ ), respectively. Bone formation in the tetracycline labelled iliac crest bone biopsy increased in the 1.0 U GH group at 3 years, but no differences were seen between the 3 groups. Body weight, height, quality of life, vertebral height on X-ray, handgrip strength and bone markers were unaltered after 3 years. One radius fracture occurred in the 1.0 U GH group. Compliance was good according to the IGF-1 levels, which were blind for the investigator during the double-blind phase. None of the 80 women dropped out. Side effects were rare. In conclusion, BMC increased up to 14% by treatment with 2.5 U GH/day during 3 years and further differentiated from the group treated with 1.0 U GH/day and the placebo group, respectively, at 4-years follow-up, in postmenopausal osteoporosis. There seems to be a delayed, extended and dose dependent effect of GH treatment on bone.

## F414

**Dermal Application of Lovastatin for 5 days Stimulates Bone Formation in Ovariectomized Rats by 160%.** G. Gutierrez,<sup>1</sup> L.R. Garrett,<sup>1</sup> G. Rossini,<sup>1</sup> A. Escobedo,<sup>1</sup> D. Horn,<sup>1</sup> M. Qiao,<sup>1</sup> J. Esparza,<sup>1</sup> D. Lalka,<sup>2</sup> G.R. Mundy.<sup>1</sup> OsteoScreen Inc., San Antonio, TX, USA, <sup>2</sup>West Virginia University, School of Pharmacy, Morgantown, WV, USA.

In recent years, it has been determined that statins, drugs which lower cholesterol by inhibiting HMG-CoA reductase, are also bone anabolic agents, causing substantial increases in bone formation in vitro and in vivo in rodents. Their effects in vitro on bone and when applied locally in vivo are more impressive than when they are administered systemically by oral gavage. Since statins are subject to first-pass metabolism by cytochrome P450 enzymes in the liver, we reasoned that dermal application may produce more sustained blood levels in the peripheral tissues such as bone, and cause more substantial effects on bone than those resulting from oral administration. We determined therefore if topical application of lovastatin to the skin of ovariectomized rats would lead to more sustained circulating blood statin concentrations and increases in bone formation rates. We used this model since it can produce alterations in the cancellous network similar to those seen in the human skeleton during aging and menopause. We measured statins in plasma by HMG-CoA reductase activity. We found that blood statin concentrations following a

single dermal dose were higher and maintained longer than following an equivalent oral dose, indicating a greater AUC after dermal application. Rats were ovariectomized and treatment with oral or topical lovastatin was started the day following surgery. In the group treated orally, lovastatin was given daily by gavage for 35 days. In the group treated dermally, the statin was applied topically (upper back after shaving) for 5 days only. Bones were analyzed after 5 weeks for both groups. Sham controls were included in both experiments. Topical lovastatin (1 mg/kg/day) caused 57% increase in trabecular bone after only 5 days of treatment. There was  $>150\%$  increase in bone formation rate, which persisted for 3 weeks without further treatment in the group treated topically. Oral lovastatin caused lesser but detectable increases in bone formation rates at a) 10 times the dose, b) after more prolonged (35 days) treatment. When Lovastatin was given orally only for 5 days, there was no effect. These results suggest that topical application of lovastatin produces greater anabolic effects on bone than oral administration and that alternative modes of administration of the statins, such as topical application through a skin patch, improves biodistribution to bone. They also show that both in vitro and in vivo, intermittent statin treatment is more effective than continuous treatment.

Disclosures: OsteoScreen, Inc., 1,3.

## F419

**The PPAR-alpha Agonist Wyeth 14643 Increases Bone Mineral Density in Female Rats.** U. Syversen,<sup>1</sup> I. Bakke,<sup>1</sup> K. W. Slørdal,<sup>2</sup> H. L. Waldum.<sup>1</sup> <sup>1</sup>Department of Intra-abdominal Diseases, Norwegian University of Science and Technology, Faculty of Medicine, Trondheim, Norway, <sup>2</sup>Department of Physiology and Bioengineering Techniques, Norwegian University of Science and Technology, Trondheim, Norway.

Peroxisome proliferator-activated receptors (PPARs) are members of the steroid nuclear superfamily of receptors that have been shown to modulate the expression of genes involved in lipid metabolism and fat storage. Recently, the presence of PPARs has also been demonstrated in bone cells and a role in bone metabolism has been postulated. In the present study we have examined the effect of the PPAR alpha agonists Wyeth 14643 and ciprofibrate on bone mineral density (BMD) in female rats. Thirty female Fischer rats were divided into 3 groups and were given methocel (control group), Wyeth 14643 (50 mg/kg body weight) and ciprofibrate (50 mg/kg body weight) for 2 months. BMD in femur and total body in intact animals was measured using a Hologic QDR 4500A. Body weight was registered throughout the study. Blood samples were drawn for measurement of gastrin. There was no difference in body weight between control rats and Wyeth 14643-treated rats, while the body weight was significantly reduced in ciprofibrate treated rats. Serum gastrin levels were significantly increased after 2 months in rats receiving ciprofibrate (x3.3) and Wyeth 14643 (x2), compared to controls. There was a significant increase in total body BMD ( $0.154 \pm 0.007 \text{ g/cm}^2$ ) in Wyeth 14643 treated rats compared to control rats ( $0.146 \pm 0.004 \text{ g/cm}^2$ ),  $p < 0.05$ , while in rats receiving ciprofibrate, total body BMD ( $0.144 \pm 0.004 \text{ g/cm}^2$ ) was significantly lower than in Wyeth 14643 treated rats. Femur BMD tended to be higher in Wyeth 14643 treated rats ( $0.263 \pm 0.011 \text{ g/cm}^2$ ) than in control rats ( $0.251 \pm 0.007 \text{ g/cm}^2$ ), and was significantly higher than in ciprofibrate treated rats ( $0.225 \pm 0.014 \text{ g/cm}^2$ ),  $p < 0.001$ . In conclusion, treatment with the PPAR-alpha agonist Wyeth 14643 significantly increased BMD in female rats, while treatment with ciprofibrate resulted in a significantly decrease in BMD.

## F422

**A Therapeutic RANKL Vaccine Induces Neutralizing Anti-RANKL Antibodies and Prevents Bone Loss in Ovariectomized Mice.** M. Hertz,<sup>1</sup> T. Juji,<sup>2</sup> S. Tanaka,<sup>2</sup> S. Mouritzen.<sup>1</sup> <sup>1</sup>M&E Biotech A/S, Hørsholm, Denmark, <sup>2</sup>Department of Orthopaedic Surgery, University of Tokyo, Tokyo, Japan.

Receptor activator of NF- $\kappa$ B ligand (RANKL) and the soluble decoy receptor osteoprotegerin (OPG) are the critical regulators of osteoclast activity. Overexpression of RANKL has been shown to be involved in the pathogenesis of resorptive bone diseases, such as osteoporosis. A therapeutic RANKL vaccine was developed by modifying the soluble TNF-like domain of murine RANKL (amino acids 158-316) to incorporate a promiscuous T helper (Th) epitope. The modified RANKL vaccine was well tolerated and able to bypass immunological tolerance and induce antibodies neutralizing native murine RANKL. Lymphoid organogenesis, lymphocyte development and activation were normal in RANKL-vaccinated mice in contrast to RANKL-deficient mice. Antiserum from vaccinated mice inhibited osteoclastogenesis in vitro. More importantly, the RANKL vaccine protected mice from ovariectomy-induced bone loss. Osteoclast numbers as well as bone resorptive surfaces were significantly reduced in RANKL vaccinated mice. Thus, vaccination against RANKL represents a novel approach for the treatment of osteoporosis, rheumatoid arthritis and other diseases associated with increased osteoclast activity and bone loss.

Disclosures: M&E Biotech A/S, 1,3.

**RANK Ligand Stimulates Anabolic Bone Formation.** J. J. Lam,<sup>1</sup> F. P. Ross,<sup>1</sup> S. L. Teitelbaum.<sup>1</sup> <sup>1</sup>Washington University School of Medicine, Saint Louis, MO, USA.

We have made the surprising observation that murine RANK ligand (RANKL), the key osteoclastogenic cytokine, when administered as an amino-terminal glutathione-S-transferase (GST) fusion protein, profoundly stimulates anabolic bone formation in vitro, ex vivo, and in vivo. Diurnal subcutaneous injection of GST-RANKL (158-316) (5 µg/kg to 4.5 mg/kg) for 7 days in mice results in a dose-dependent net increase in the number of activated osteoblasts in long bones, maximizing at 25-fold relative to control (624 ± 43 ob/mm<sup>2</sup> experimental vs. 28 ± 4 ob/mm<sup>2</sup> control,  $p < 0.001$ ). In contrast, GST-RANKL fails to affect osteoclast number (45 ± 10 oc/mm<sup>2</sup> experimental vs. 39 ± 5 oc/mm<sup>2</sup> control). Systemic administration of GST-RANKL for 7 days induces as much as a 3-fold increase in cortical bone thickness, structural augmentation of the microarchitecture of the primary spongiosa, and a 10% increase ( $p < 0.01$ ) in bone mineral density by DEXA. Dual fluorochrome labeling in animals receiving 1.5 mg/kg/day GST-RANKL for 7 days demonstrates a mineral apposition rate (MAR) of  $5.59 \pm 0.18$  µm/day in the parietal bones of GST-RANKL-treated animals, compared with  $0.18 \pm 0.03$  µm/day in the parietal bones of control animals, ( $p < 0.001$ ). Marrow derived from mice administered 1.5 mg/kg/day GST-RANKL for 14 days exhibits a 100-fold increase in mineralizing bone nodules, when cultured under osteoblastogenic conditions ex vivo. Furthermore, administration of GST-RANKL for 12 hours, given on days 1 and 4 of ex vivo whole organ culture, induces a dose-dependent increase in the thickness of calvaria, maximizing at 2-fold ( $46.1 \pm 3.97$  µm experimental vs.  $20.7 \pm 1.5$  µm control,  $p < 0.001$ ). Consistent with the osteogenic properties of GST-RANKL, we find that its receptor RANK is expressed by primary mesenchymal osteoprogenitor cells, and that these cells respond to GST-RANKL with activation of the NFκB and MAPK pathways. Attesting to the function of RANKL-RANK signaling in osteoprogenitor cells, expression of the osteoblast transcription factor Cbfa1 is enhanced within 1 hour of in vivo treatment with GST-RANKL. Thus, a chimeric derivative of the pro-resorptive cytokine RANKL can induce bone formation by a mechanism involving RANK signaling in early osteoblast precursors to enhance Cbfa1 expression and induce commitment to the osteogenic phenotype. RANKL, or derivatives thereof, therefore presents itself as a potential anabolic agent for bone.

## 1054

**Cells of the Osteoblastic Lineage from Transgenic Mice Over-Expressing Cbfa1 Induce Increased Bone Resorption in Vitro.** V. Geoffroy,<sup>1</sup> M. Kneissel,<sup>2</sup> B. Fournier,<sup>2</sup> P. Mathias.<sup>1</sup> <sup>1</sup>Friedrich Miescher-Institute, Basel, Switzerland, <sup>2</sup>Bone Pharma, Novartis, Basel, Switzerland.

The transcription factor Cbfa1 is required for bone formation. It acts as a differentiation factor during mesenchymal condensation and is also important for proper osteoblastic function, but its role in adult bone remodeling is not fully understood. To address this question we generated transgenic mice overexpressing Cbfa1 under the control of the rat collagen type I promoter. These mice present a severe osteopenic phenotype associated with high bone turn-over, cortical bone loss, and multiple fractures. To understand the origin of the increased bone resorption, we developed bone marrow stromal cell cultures and reciprocal co-culture of primary osteoblasts and spleen cells from wild type and transgenic mice followed by TRAP staining. The culture of bone marrow stromal cells under osteoclastogenic conditions showed that stromal cells of transgenic genotype induced an increased number of TRAP positive multinucleated cells compared to wild-type cells. To confirm these results we performed co-culture experiments from osteoblasts derived from calvaria and spleen cells. As expected primary osteoblasts derived from transgenic mice trigger the generation of more TRAP positive osteoclastic cells suggesting that primary osteoblasts as well as bone marrow stromal cells from transgenic mice have stronger osteoclastogenic properties compared to cells derived from wild-type animals. We then investigated the candidate genes that could trigger this increase of TRAP positive osteoclasts and analyzed the expression of bone markers in calvaria-derived cells and stromal cells isolated from wild type and transgenic animals. In these cells, we showed by semi-quantitative RT-PCR experiments, that the transgene was expressed in both cell populations and that the expression of RANKL and collagenase 3, two factors involved in formation-resorption coupling, were markedly increased in transgenic cells. These results are consistent with an increase of these two factors measured in RNA prepared from long bone of transgenic animals. Our data rather suggest that the overexpression of Cbfa1 in cells of the osteoblastic lineage, including bone marrow stromal cells, have a positive effect on osteoclast differentiation and consequently on bone resorption. These findings also indicate that the increase of bone turn over observed in the mice overexpressing Cbfa1 under the control of the collagen type I promoter is partly due to the high expression level of RANKL and collagenase 3 in bone marrow stromal cells.

## 1055

**Longitudinal Transmenopausal Changes in Three-Dimensional Trabecular Microarchitecture and Connectivity of Human Iliac Crest Bone Biopsies.** Y. Jiang,<sup>1</sup> J. Zhao,<sup>1</sup> R. R. Recker,<sup>2</sup> M. W. Draper,<sup>3</sup> H. K. Genant.<sup>1</sup> <sup>1</sup>Osteoporosis and Arthritis Research Group, University of California, San Francisco, CA, USA, <sup>2</sup>Osteoporosis Research Center, Creighton University, Omaha, NE, USA, <sup>3</sup>Lilly Research Laboratories, Indianapolis, IN, USA.

This study was designed to capture true longitudinal transmenopausal changes in three-dimensional (3D) trabecular architecture, which may improve our ability to understand the pathophysiology of osteoporosis and other bone disorders, and to estimate bone biomechanical properties in terms of fracture resistance as the mechanical competence of trabecular bone is a function of its apparent density and 3D distribution. During aging and diseases such as osteoporosis, trabecular plates are perforated and connecting rods are dissolved, with a continuous shift from one structural type to the other. Such changes can not be evaluated by 2D histological sections. In histomorphometry, there is debate about whether trabecular thinning occurs, or rather trabecular disappearance occurs with aging and/or menopause based on 2D sections using the parallel plate model. We examined paired bone biopsies from the iliac crest, not a primary weight bearing anatomical site, from 20 Caucasian women. The first biopsy was from normal, premenopausal women, age 46.3 ± 53.5 years (mean ± SD, 49.1 ± 2.7 years), and the second biopsy from the same group of women, but 12 months postmenopausal, occurring 2.3 – 8.4 years ( $5.0 \pm 1.7$  years) after the first biopsy. The specimens were scanned using a micro computed tomography scanner (µCT 20, Scanco) with isotropic resolution of 20 µm. 3D trabecular structural parameters were directly measured without stereological model assumption. Values of 0 and 3 for the structure model index represent an ideal plate structure and rod structure, respectively, while values ranging from 0 to 3 indicate a structure with both plates and rods of equal thickness, depending on the volume ratio of rods and plates. After menopause, there was a significant change in 3D trabecular bone volume fraction (-5.4%/yr), trabecular number (-1.2%/yr), trabecular thickness (-3.3%/yr), trabecular separation (+2.0%/yr), structure model index (+11.3%/yr), degree of anisotropy (-0.7%/yr), and connectivity density (-2.0%/yr). The percentage change over the mean 5-year period was greater in 3D trabecular thickness (-16.4%) than in trabecular number (-6.1%) and trabecular separation (+9.9%). Thus, there is a rapid deterioration of 3D trabecular structure and connectivity in the iliac crest in the initial postmenopausal year. Trabecular thinning does occur and trabeculae dramatically shift from a plate-like structural type to a rod-like pattern, and become more isotropic.

## 1056

**A Novel Therapeutic Vaccine That Prevents Pathological Bone Destruction in Models of Osteoporosis and RA.** T. Juji,<sup>1</sup> K. Aoki,<sup>2</sup> D. Horie,<sup>2</sup> K. Ohya,<sup>2</sup> M. Herz,<sup>3</sup> A. Gautam,<sup>3</sup> S. Mouritsen,<sup>3</sup> H. Oda,<sup>1</sup> K. Nakamura,<sup>1</sup> S. Tanaka.<sup>1</sup> <sup>1</sup>Department of Orthopaedic Surgery, Faculty of Medicine, The University of Tokyo, Tokyo, Japan, <sup>2</sup>Section of Pharmacology, Department of Hard Tissue Engineering, Graduate school, Tokyo Medical and Dental University, Tokyo, Japan, <sup>3</sup>M & E Biotech A/S, Hørsholm, Denmark.

The receptor activator of NF-κappaB ligand (RANKL) is a novel member of tumor necrosis factor family cytokines, which is critically involved in osteoclast differentiation and activation, and therefore important for normal bone development. There is accumulating evidence that RANKL also plays important roles in the pathological bone destruction such as osteoporosis and rheumatoid arthritis. The natural inhibitor of RANKL, osteoprotegerin (OPG), has potent therapeutic effects on such conditions. However, repeated administration of OPG in larger doses could potentially be immunogenic and may elicit antibody responses, limiting its long-term effectiveness. Here we describe a simple and effective method of active immunization against self RANKL as a possible treatment of bone diseases. RANKL protein vaccines were generated by inserting a promiscuous foreign T helper (Th) peptide into the RANKL cDNA. Immunization with these vaccines resulted in a rapid and sustainable polyclonal anti-RANKL antibodies in mice. No apparent macroscopic abnormality was observed in any organs of the immunized animals. To determine the therapeutic effects of these vaccines, we utilized ovariectomy and arthritis models. Female BALB/c mice were immunized with either control antigen or the Th peptide-modified RANKL vaccine four times at two week intervals before subjecting them to ovariectomy. Mice immunized with RANKL vaccines were resistant to bone loss in response to ovariectomy. Importantly, both osteoclast numbers as well as bone resorption surface were significantly reduced in RANKL vaccinated mice. We next examined the effect of the vaccine on SKG mice, a natural mutant of BALB/c background, that develops spontaneous rheumatoid arthritis-like inflammatory joint disorders and bone destruction. Immunization with RANKL vaccines almost completely prevented the bone destruction in these mice. Osteoclast numbers in various regions of bones were also dramatically reduced following this vaccination. These results demonstrate that a therapeutic vaccine approach targeting RANKL can be used to inhibit bone destruction in a variety of pathological bone loss conditions such as osteoporosis and rheumatoid arthritis.